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Novel signaling pathways in ischemic and hypertensive renal injury

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von

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Abbreviations

AKI: Acute Kidney Injury

Ang II: Angiotensin II

AT1R: Angiotensin II Type 1 Receptor

Bcl10: B cell lymphoma/leukemia 10

BP: Blood Pressure

CARMA1/3: Caspase Recruitment Domain-Containing MAGUK protein 1/3

CBM: CARMA1/3-Bcl10-MALT1

CD: Cluster of Differentiation

CKD: Chronic Kidney Disease

cKO: conditional Knockout

CTH/Cth: Cystathionase protein/gene

DNA: Deoxyribonucleic Acid

eGFR: estimated Glomerular Filtration Rate

ESRD: End-stage Renal Disease

FSGS: Focal Segmental Glomerulosclerosis

IFNγ: Interferon-gamma

IFNγR: interferon-gamma Receptor

IKK: IκB kinase complex

IL: Interleukin

IL23R: Interleukin 23 Receptor (IL23R)

IRI: Ischemia Reperfusion Injury

IκB: Inhibitor of NF-κB

KO: Knockout

loxP: locus of x-over, P1

MALT1: Mucosa-associated lymphoid tissue lymphoma translocation protein 1

NF-κB: Nuclear Factor kappa-light-chain-enhancer of activated B cells

RAAS: Renin-Angiotensin-Aldosterone system

RNA: Ribonucleic Acid

mRNA: messenger Ribonucleic Acid

TGFβ: Transforming Growth Factor-beta

TNFα: Tumor Necrosis Factor-alpha

TRPC6: Transient Receptor Potential, Canonical 6

I. Introduction

Whereas the kidneys as organs were recognized already in ancient Egypt[1] and one of the most accomplished medical researchers of the Roman Empire, Claudius Galenus, described the primary function of the kidneys (i.e., urine generation) correctly,[2] it was not until the discovery of the microscope and the work of Marcello Malpighi in the seventeenth century when the microscopic anatomy of the kidney and the hypothesis was born that formation of urine takes place in the kidneys through a filtering mechanism between blood and renal tubules.[3] After three centuries and many bright minds later, such as Richard Bright, Theodor Fahr, and Franz Volhard, to name a few. The next milestone marked the development of electron microscopy, which coincided with the introduction of renal biopsy and the establishment of experimental renal pathology.[3] Whereas the understanding of the pathophysiology of renal diseases has been progressing ever since at an unprecedented pace, the clinical subspecialty of nephrology emerged only some 60 years ago when transplantation and dialysis became available in the 1960s.[4] Although these groundbreaking interventions have made it possible to save and prolong the lives of patients with "end-stage renal disease", recognizing the development stages of this "end-stage renal disease" lagged. It was not earlier as in 2002 when the first uniform definition - based on objective measures of kidney function - staging system for chronic kidney disease (CKD) was developed and 2004 for acute kidney injury (AKI), which was named at this time "acute renal failure".[5,6] Once definitions were established, it became clear that AKI, as well as CKD, have both high prevalence and associated with increased mortality and morbidity. AKI can be diagnosed in around 10–15% of hospitalized patients, while this number can be as high as 50% in intensive car units.[7,8] The exact figures apply for CKD: population prevalence is more than 10% and exceeds 50% in subpopulations with conditions posing a high risk of developing CKD.[9-11]

Clinical diagnosis of AKI is still based on rapid changes in creatinine levels in the blood and reduced urine volume. Stage 1 AKI refers to a creatinine elevation in the blood ≥1.5 times of baseline value or an increase of ≥0.3 mg/dL in two days or a urine volume <0.5 mL/kg for 6–12 hours. Stage 2 refers to a blood creatinine two times or more of the baseline value or to a urine volume <0.5 mL/kg for ≥12 h. Lastly, stage 3 refers to a blood creatinine ≥3.0 times of the baseline concentration or an increase to ≥4.0 mg/dL or acute dialysis, or urine volume <0.3 mL/kg for ≥24 h.[12] CKD is defined as (estimated) glomerular filtration rate (e)(GFR)<60 mL/min per 1.73 m² and/or presence of renal damage markers - such as albuminuria (urine albumin to urine creatinine ratio>30mg/g), abnormal urinary sediment, electrolyte or other

abnormality due to renal tubular disorder, abnormal renal histology or history of renal transplantation for at least 3 months. CKD is clinically classified according to international into the following categories based on (e)GFR (mL/min per 1.73 m²) as follows: G1 ≥90 (normal or high), G2 60–89 (mildly reduced), G3a 45–59 (mildly to moderately reduced), G3b 30–44 (moderately to severely reduced), G4 15–29 (severely reduced) and G5 <15 (kidney failure).[13] Although the clinical diagnosis of AKI and CKD can be made based on these set values and provide helpful guidance in treating these conditions, the underlying pathophysiology varies, and many cases are poorly understood.[12] In fact, treatment of AKI and CKD in almost all cases is limited to supportive treatment or ameliorating complications and consequences of renal function loss.

AKI induced by prerenal factors and leading to reduced blood flow is the most common form of AKI regarding the number of hospitalized patients. Hypoxia, followed by reperfusion injury, is a major pathophysiological driver of renal damage, especially in multiple organ failure and sepsis.[14,15] What we nowadays know about the pathophysiological processes of prerenal AKI is derived mainly from animal models, especially acute ischemia models induced by temporal clamping of the renal vessels (the renal artery only or in mouse models commonly clamping both the renal artery and vein) leading to ischemia-reperfusion injury of the kidney.[16-20] After a reduction in adequate kidney perfusion, virtually all kidney cell types are affected, but more severely, cells with high metabolic rates and limited capacity to switch to anaerobic glycolysis. This involves the proximal tubular cells (mainly the straight (descending) portion of the proximal tubule) and tubular epithelial cells of the medulla, which are – due to the countercurrent capillary flow – in a healthy situation already in a "physiological" hypoxic state.[21] In these epithelial cells, hypoxia or ischemia leads to depletion of adenosine triphosphate and consequent cell injury, which may ultimately lead to programmed cell death (apoptosis) or even necrosis.[15,22] However, tubular epithelial cells not only suffer from morphological, cytoskeletal, and structural changes (or even cell death) but are also active contributors to the repair. The surviving tubular cells undergo dedifferentiation and proliferation to replace dead tubular cells and also actively produce cytokines and chemokines that further define the degree of cellular infiltration and regulate innate and adaptive immune responses.[15,23] Cytokines and chemokines produced by tubular cells include interleukin (IL)6, IL1β, chemokine (C-C motif) ligand 2, monocyte chemoattractant protein 1, tumor necrosis factor (TNF)α and transforming growth factor (TGF)β.[15,22] Many of these molecules are controlled by the transcription factor family nuclear factor kappa-light-chain-enhancer of activated B cells (NF-κB).[24,25] The mammalian NF-κB family consists of five proteins that can form several homo- or heterodimers. These members are called NF-κB1 (p105/p50), NF-κB2

(p100/p52), RelA (p65), RelB and c-Rel. The denotation "NF-κB" commonly refers to the p50-RelA heterodimer (used in this work also in this sense), which is the major complex in most cells.[26] Indeed, NF-κB regulates several aspects of the adaptive and the innate immune systems, thereby mediating inflammatory responses and regulating the activation, survival, and differentiation of immune cells and other cell types, including renal cells.[24,25] To analyze the time course of NF-κB activation after ischemic renal injury and assess the role of tubular NF-κB in post-ischemic cellular infiltration and chemokine and cytokine expression-specific mice models were developed and applied, which are described in the first original article of this work.

A further hallmark of (ischemic) renal damage is endothelial dysfunction. Under physiological circumstances, endothelial cells contribute to vascular tone, thereby regulating the blood flow of the local tissue beds. Modulation of vascular permeability regulates cellular infiltration into the interstitium. Damaged endothelium has increased microvascular permeability and has an imbalance between vasoconstrictor and vasodilator agents, with nitric oxide as the central mediator.[15,22,27] Next to nitric oxide and carbon monoxide, hydrogen sulfide has emerged recently as a novel gaseous mediator in renal tissue.[28,29] Whereas the application of extrinsic hydrogen sulfide showed potential to ameliorate acute ischemic renal damage[30,31], the role of the endogenously produced hydrogen sulfide was unclear. Endogenous hydrogen sulfide in the kidney is mainly produced by cystathionine gamma-lyase and by cystathionine beta-synthase. In the second original article, we investigated the role of endogenous hydrogen sulfide by using mice lacking or having only one or both copies of the cystathionine gamma-lyase gene.

Once the kidney suffers insult, intrinsic repair processes are activated rapidly. This process can lead to (complete or partial) resolution by adaptive repair mechanism or progression by maladaptive repair mechanism.[32] Maladaptive repair can occur in the tubular (epithelial), vascular, and interstitial compartments of the kidney in response to insult, ultimately leading to renal fibrosis and reduction in kidney function.[23] Glomeruli scarring secondary to podocyte damage, a critical cell type of the ultrafiltration barrier, can lead to focal segmental glomerulosclerosis (FSGS), ultimately leading to CKD. Such podocyte damage can be caused by identifiable factors (considered as the primary form of the disease) and the secondary form of the disease by toxins, drugs, or are associated with viral infection or mutations in differential genes.[33] Mutations of the transient receptor potential cation (TRPC) channel 6 – a member of non-selective Ca^{2+}-permeable TRPC channels – have been found in families with FSGS.[34] Subsequent studies have shown that the TRPC6 channel is expressed in podocytes and represents a component of the slit diaphragm. This specialized cell junction comprises several

cell-surface proteins and connects neighboring podocyte foot processes.[35,36] Additionally, TRPC6 channels are expressed in other renal structures, such as glomerular mesangial cells, tubular epithelial cells, and other cells of renal microvasculature. More importantly, the TRPC6 channels are also redox-sensitive.[37] An imbalance of redox signaling is a hallmark of AKI.[38] More recently, treatment with BI-749327, a potent and selective orally bioavailable TRPC6 antagonist, or having TRPC6 genetically knocked out, has been found to ameliorate renal fibrosis in the model of unilateral ureteral obstruction.[39,40] Based on these findings, we hypothesized that inhibition of TRPC6 might be helpful to alleviate the immediate outcome of AKI. To address this hypothesis, we used mice lacking the gene of TRPC6 or treated wild-type mice who underwent AKI induction by BI-749327 or by SH045, a further selective inhibitor of TRPC6. The third article describes the usefulness of targeting TRPC6 to impact the outcome of ischemic AKI.

High blood pressure (hypertension) is the main cause of cardiovascular disease and premature death worldwide.[41] In human progressive chronic kidney damage, hypertension is the second leading cause of end-stage renal disease[42] and a significant risk factor for developing chronic kidney disease.[43,44] On the other hand, a common consequence of chronic kidney disease is hypertension.[45] Therefore, the interaction between hypertension and (chronic) kidney damage is complex. Several animal models were developed and analyzed in the last decade to disentangle the relationship between renal damage and hypertension and to understand the pathogenesis of hypertension.[46] One of the most widely used models is the long-term (at least 4 weeks) angiotensin (Ang) II-induced hypertension model because it closely resembles some aspects of human hypertension and target-organ damage, including vascular remodeling, cardiac hypertrophy, and chronic kidney disease.[46] Using this (and also verified in other models), the concept that immune cells contribute to hypertension has been established.[47-49] With the advancing technology of genetically engineered mouse models, mice lacking the recombinase activating gene 1 were generated. The protein encoded by this gene is involved in immunoglobulin and T-cell receptor recombination; therefore, mice lacking this gene fail to develop B and T lymphocytes. In a milestone paper, mice lacking this gene develop blunted hypertension in response to angiotensin II or deoxycorticosterone acetate salt (another agent to induce hypertension in murine models)[50] challenge. Adoptive transfer of T (but not B) cells into these mice leads to hypertension and end-organ damage.[51] Interferon-gamma (IFNγ) is an important cytokine that regulates the host defense system by interacting with innate and adaptive immune systems. Its signaling pathway coordinates a diverse array of cellular programs.[52,53] In the fourth manuscript, the role of this pathway was investigated in the development of hypertensive organ damage with particular emphasis on renal damage.

As outlined earlier, the transcription factor family NF-κB controls many normal cellular processes by regulating the expression of genes and proteins involved in immune system homeostasis, inflammatory responses, cellular growth, or apoptosis. Ang II can activate NF-κB through its angiotensin II type 1 (AT1R) and type II receptor.[54,55] More importantly, NF-κB inhibition has been shown to ameliorate Ang II-induced renal damage.[56] Nevertheless, the molecular signaling between the AT1R and NF-κB was unknown. It was not until 2007 that McAllister-Lucas and colleagues described a signalosome filling the signaling gap between AT1R and NF-κB. In collaboration with this group and by performing bone-marrow transplantation studies with mice lacking the intermediate bridging factor of the signalosome mentioned above, we could shed light on its role in immune cells and renal tissue in an Ang II-induced hypertension model.

In summary, my experimental works aimed to identify novel pathways in the kidney which influence the outcome of AKI and are involved in hypertension-induced progressive renal damage, with the hope to provide molecular targets and, therefore, specific therapy options for the future.

II. Original articles

1. Tubular Epithelial NF-kB Activity Regulates Ischemic AKI

Markó L*, Vigolo E*, Hinze C, Park JK, Roël G, Balogh A, Choi M, Wübken A, Cording J, Blasig IE, Luft FC, Scheidereit C, Schmidt-Ott K, Schmidt-Ullrich R, Müller DN. J Am Soc Nephrol. 2016;27:2658-2669.
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Acute kidney injury (AKI) is a clinical syndrome characterized by a rapid (hours to days) reduction or loss of the kidney's excretory function, leading to the retention of waste products in the blood, such as creatinine and urea.[14] It is a common complication, affecting 10-15% of all hospitalized patients.[7] In the case of particular patient groups - such as patients in intensive care units – the prevalence can be more than 50%.[8] Traditionally, AKI is classified based on the anatomical site of the injury to prerenal, intrinsic, and postrenal AKI.[12] Whereas clinically many conditions can lead to AKI, the pathophysiological mechanisms of AKI are less clear, and the mechanisms are still poorly understood; increasing evidence suggests that each clinical entity has a unique pathophysiology.[12] Still, hypoperfusion and consequent ischemic injury are considered to be one of the initiator factors of renal damage in many forms of AKI.[12]

The understanding of the molecular mechanisms behind (prerenal) AKI is mainly derived from animal models of acute ischemia induced by temporal occlusion of the renal artery, which provokes renal ischemia-reperfusion injury (IRI). Mechanisms identified by these studies include the local activation of the coagulation system[57], infiltration of immune cells[58], damage of the endothelial cells[59], and expression of adhesion molecules[60] and cytokines[61] or activation of endogenous danger molecule receptors such as the toll-like receptor-4.[62]

Probably NF-κB is one of (if not the most) studied transcription factor family consisting of the protein p50/p105 (NF-κB1), p52/p100 (NF-κB2), p65 (RelA), c-Rel, and RelB. These can form various heterodimers or homodimers involved in multiple cellular processes, such as modulating the immune system's activity and inflammatory responses and regulating cell growth and apoptosis.[63] The p65-p50 heterodimer (referred to as NF-κB in this work) is activated by a wide range of stimuli relevant to kidney injury, including inflammatory

molecules, chemokines, growth factors, and toll-like receptors.[64] In unstimulated cells, NF-kB is retained in the cytoplasm through interaction with NF-κB inhibitor proteins (IκBs). Upon specific stimuli, the IκB kinase complex (IKK) phosphorylates the IκB proteins, leading to its ubiquitination and degradation, allowing translocation of NF-κB to the nucleus.[65]

The pleiotropic effect of NF-κB strongly implies that its modulation could influence the course of IRI and the outcome of AKI. The first studies applied an NF-κB decoy strategy in rat kidney allograft transplantation[66] and rat renal IRI models[67] or administrated systemically small interfering ribonucleic acids (RNA) targeting RelB[68] or IκB kinase subunit b[69] in animal models of AKI. Whereas these studies could show that general NF-kB inhibition attenuates renal cellular infiltration and, subsequently, IRI, they did not shed light on the role of NF-κB in the renal tissue.

In our study, we applied a novel transgenic mouse model expressing the luciferase gene under the control of NF-κB. In mice, renal IRI was achieved by uninephrectomy and temporal clamping of the remaining renal pedicle. With this mouse model's help and *in vivo* bioluminescence imaging technology, we could assess the time course of NF-κB activation of an IRI-induced AKI. The NF-κB activation in the kidney increased significantly as soon as 12 hours after renal ischemia, peaked 2-3 days, and stayed elevated for up to 5 days compared to sham-operated littermate mice. Immunostaining against phospho-S276-p65 (an indicator of nuclear translocation) showed that NF-κB was mainly activated in renal tubular cells, suggesting that the detected signal was derived from these cells rather than from infiltrating immune cells. Using the Cre-loxP technology, – where Cre is a recombinase enzyme that recognizes the specific deoxyribonucleic acid (DNA) fragment sequences called loxP (locus of x-over, P1)[70] –, we expressed IκBαΔN specifically in renal tubular cells. IκBαΔN is a genetically engineered IκBα that lacks the N-terminal phosphorylation and ubiquitination sites and thus precludes nuclear translocation of NF-κB). To express this construct tubular cell-specific, we mated Emx1-Cre and loxP-IκBαΔN mice to get mice with lower renal tubular NF-κB activity, referred further as Emx1-ΔN. Twenty-four hours after IRI-induced AKI, Emx1-ΔN mice developed less renal epithelial damage, had less neutrophil and macrophage infiltration in the kidney tissue, and expressed lower levels of adhesion molecules, proinflammatory cytokines, and chemokines in comparison to control littermates (mice expressing the Cre enzyme only, the floxed IκBαΔN construct or none of these) which underwent the same surgical procedure, verifying earlier findings with systemic NF-κB inhibition strategies.[66-69] More importantly, Emx1-ΔN mice after renal ischemic injury had lower serum creatinine concentrations and expression of renal damage marker neutrophil gelatinase-associated lipocalin, indicating a clinically relevant amelioration of renal damage. Using microarray data of renal tissue of mice that

underwent IRI-induced AKI at time points, we observed NF-κB activation (6, 24, and 48 hours and 7 days) and clustered the time-dependent expression pattern of all genes which were downregulated in injured kidneys of Emx1-ΔN mice compared to littermate controls. We limited ourselves to the analysis of these genes because, in a priori gene-set enrichment analysis, they showed enrichment for known NF-κB targets. The first cluster contained genes primarily involved in (programmed) cell death and chemotaxis. To further analyze this process, we performed terminal deoxynucleotidyl transferase-mediated digoxigenin-deoxyuridine nick-end labeling to detect DNA fragmentation resulting from apoptosis. Interestingly, the IRI-injured renal tissue of Emx1-ΔN mice showed fewer apoptotic cells than the IRI-injured control renal tissue. Mimicking hypoxia *in vitro* using cobalt chloride containing low-glucose medium in primary proximal tubular cells isolated from Emx1-ΔN and littermate wild-type mice verified the reduced apoptotic rate in Emx1-ΔN cells.

In summary, we determined the *in vivo* time course of NF-κB activation and shed light on its function in the renal tubular epithelial cells during a renal IRI. We found that postischemic NF-κB activation in the renal tubular epithelia worsens tubular injury, inflammatory response, and apoptosis.

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2. Role of Cystathionine Gamma-Lyase in Immediate Renal Impairment and Inflammatory Response in Acute Ischemic Kidney Injury

Markó L, Szijártó IA, Filipovic MR, Kaßmann M, Balogh A, Park JK, Przybyl L, N'diaye G, Krämer S, Anders J, Ishii I, Müller DN, Gollasch M. Sci Rep. 2016;6:27517.

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Gaseous signaling molecules play an essential role in various biological processes in the human body. Next to nitric oxide and carbon monoxide, hydrogen sulfide (H_2S) emerged in the last decades as the third physiological gaseous mediator by measuring endogenous concentrations in brain homogenate of rats.[71,72] Soon, it was recognized as an endogenous neuromodulator[73], a vasorelaxant[74], and later even identified as garlic's cardioprotective signaling molecule.[75] In mammalian systems, H_2S is produced by two enzymes: cystathionine β-synthase and cystathionine γ-lyase (CTH, also referred to as cystathionase or CSE); the former is responsible for H_2S production in the brain and later in the vascular system.[76] The physiological role in H_2S production of the third enzyme, 3-mercaptopyruvate sulfurtransferase, is not entirely cleared yet, although, in a recent paper, this enzyme was found to be the primary source of H_2S in coronary arteries.[77]

In the vascular system, H_2S causes vasorelaxation by activating adenosine triphosphate-sensitive potassium channel channels on smooth muscle cells.[74] Additionally, H2S can act as an antioxidant by elevating intracellular glutathione concentration and increasing the levels of the glutathione biosynthetic enzyme.[78] These properties of H_2S make it an excellent potential therapeutics, especially in diseases involving ischemic injury. Soon the cardio[79]- and neuroprotective effects[80], as well as reno-[31,81], hepato[82]- and pulmoprotective[83] effects of H_2S were established.

At that time, all animal studies applied H_2S or H_2S donor molecules or used pharmacologic inhibitors of H_2S-producing enzymes to investigate the role of H_2S in disease, as no knockout animal models existed. However, the physiological concentrations of H_2S in tissues have been debated since its discovery because of methodological difficulties in its measurement.[84,85] Consequently, a wide range of values for H_2S have been reported in blood or tissues. This state of affairs generated significant uncertainty in the scientific community. Knockout mice

have been developed in two laboratories to overcome this issue and asses the (patho)physiological functions of endogenous H_2S.[86,87] Although in both laboratories Cth was targeted to knock out (though different exons were deleted and somewhat different techniques were used during knockout mouse generation), surprisingly, the two mouse models showed some important differences - next to shared properties. For example, knockout mice from the laboratory of Rui Wang developed age-dependent blood pressure elevation[87]. In contrast, the other one from the laboratory of Ishao Ishii[86] had similar or even slightly lower blood pressure[88] compared to littermate wild-type mice.

In our study, we investigated the role of endogenous H_2S in ischemic renal disease. We used the mouse model of Ishii et al.[86] We mated $Cth^{+/-}$ males and females to obtain $Cth^{+/+}$, $Cth^{+/-}$, and $Cth^{-/-}$ littermates. First, we confirmed on both messenger ribonucleic acid (mRNA) and protein levels that $Cth^{-/-}$ animals do not express cystathionase. Heterozygous animals had expression levels half of the wild-type, $Cth^{+/+}$ animals. However, we still measured approximately 50% and 30% of H2S content in the renal tissue of $Cth^{+/-}$ and $Cth^{-/-}$ mice, respectively, compared to $Cth^{+/+}$ littermates, strongly suggesting that CTH is not the source of H_2S in the kidney. Mice underwent the same IRI protocol as described earlier.[89] Ischemia significantly reduced the expression of all three H_2S-producing enzymes in the kidney, in agreement with previous reports.[90] After induction of ischemic AKI in $Cth^{+/+}$, $Cth^{+/-}$, and $Cth^{-/-}$ mice, serum creatinine concentrations and expression of kidney injury molecule 1 and renal damage marker neutrophil gelatinase-associated lipocalin were analyzed 24 hours after injury. Unexpectedly, we could not detect significant differences between the 3 experimental mice groups; moreover, $Cth^{-/-}$ mice had the lowest serum creatine levels. Histopathological analysis of tubular damage showed a similar trend, as well as mRNA expression analysis of cytokines and adhesion molecules in the injured renal tissue. Although flow cytometric analysis of infiltrating neutrophils and macrophages did not reveal differences between the 3 groups, the lower renal expression of IL1-beta and TNFα in injured kidneys of $Cth^{-/-}$ mice led us to test the hypothesis that Cth deficiency affects these cytokines producing[91] classically activated macrophages. We polarized *in vitro* bone marrow-derived macrophages of $Cth^{+/+}$ and $Cth^{-/-}$ mice using lipopolysaccharide and interferon-gamma. Whereas the mRNA levels of TNFα in polarized $Cth^{-/-}$ macrophages were significantly lower compared to the polarized $Cth^{+/+}$ macrophages, the concentration of TNFα in the supernatant of the $Cth^{+/+}$ and $Cth^{-/-}$ macrophages was comparable.

We concluded that lacking CTH leads to approximately a 50% reduction in renal H_2S levels in mice. However, this does not significantly affect the immediate outcome of ischemic AKI. Of note, while we were performing the experiments, Bos et al. published findings with renal

ischemia using the other existing Cth knockout mouse model.[92] They observed over 90% reduction in renal H_2S production in CTH deficient mice and lethal renal injury (35% mortality) with excessive tubular damage compared to wild-type mice. This phenotype was associated with more oxidative damage and could be rescued by intraperitoneal injection of H_2S donor NaHS. The different outcomes of renal IRI in the study of Bos et al. and ours are probably multifaceted, which at least in part anchors in the fundamental phenotypic differences of the two mouse models. Therefore, further studies are needed to establish the role of endogenous H_2S in renal pathophysiology.

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Role of Cystathionine Gamma-Lyase in Immediate Renal Impairment and Inflammatory Response in Acute Ischemic Kidney Injury

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Hydrogen sulfide (H_2S) is known to act protectively during renal ischemia/reperfusion injury (IRI). However, the role of the endogenous H_2S in acute kidney injury (AKI) is largely unclear. Here, we analyzed the role of cystathionine gamma-lyase (CTH) in acute renal IRI using CTH-deficient ($Cth^{-/-}$) mice whose renal H_2S levels were approximately 50% of control (wild-type) mice. Although levels of serum creatinine and renal expression of AKI marker proteins were equivalent between $Cth^{-/-}$ and control mice, histological analysis revealed that IRI caused less renal tubular damage in $Cth^{-/-}$ mice. Flow cytometric analysis revealed that renal population of infiltrated granulocytes/macrophages was equivalent in these mice. However, renal expression levels of certain inflammatory cytokines/adhesion molecules believed to play a role in IRI were found to be lower after IRI only in $Cth^{-/-}$ mice. Our results indicate that the systemic CTH loss does not deteriorate but rather ameliorates the immediate AKI outcome probably due to reduced inflammatory responses in the kidney. The renal expression of CTH and other H_2S-producing enzymes was markedly suppressed after IRI, which could be an integrated adaptive response for renal cell protection.

Hydrogen sulfide (H_2S) has been recognized as a toxic gas for many years until Warenycia *et al.* discovered the endogenous production of H_2S in the rat brain[1]. Endogenous H_2S is mainly produced by enzymes of the transsulfuration pathway, cystathionine gamma-lyase (CTH or CSE) and cystathionine beta-synthase (CBS). The third enzyme, 3-mercaptopyruvate sulfurtransferase (MPST or MST), can also contribute to endogenous H_2S production in the presence of reductants using 3-mercaptopyruvate as a substrate[2–4]. Although the physiological role of MPST in mammalian tissues is less well characterized, MPST might contribute to H_2S production in the brain or modulation of cardiovascular functions[5,6].

Several studies demonstrated that H_2S could exert protective effects in the cardiovascular system. In particular, H_2S has emerged as potential therapeutics[3] for ischemia/reperfusion injury (IRI) of different organs[2]. This knowledge mainly relies on the results from animal studies using H_2S/H_2S donor molecules or pharmacological inhibitors of H_2S-producing enzymes[7]. However, the physiological levels of H_2S in tissues have been a matter of debate because of methodological difficulties in measuring accurate, reliable, and reproducible H_2S levels in

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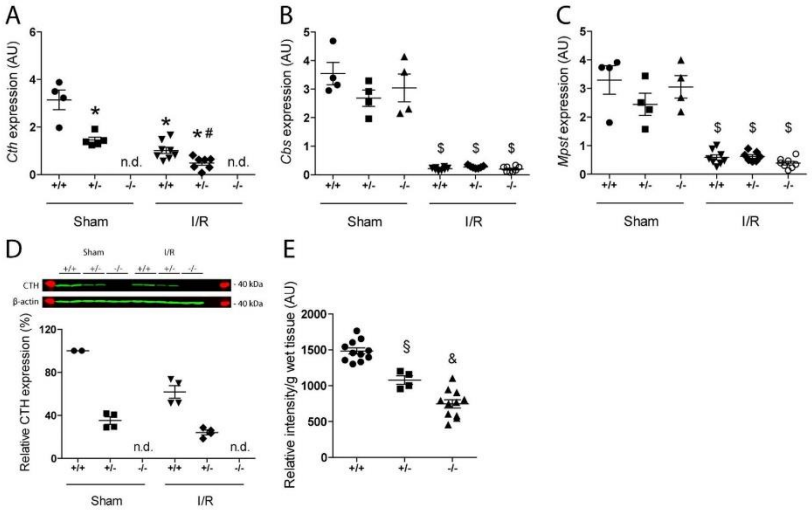

Figure 1. Renal expression of H_2S-producing enzymes and H_2S. Gene expression levels of (**A**) cystathionine gamma-lyase (*Cth*), (**B**) cystathionine beta-synthase (*Cbs*) and (**C**) 3-mercaptopyruvate sulfurtransferase (*Mpst*) in sham- and ischemia/reperfusion (I/R)-injured kidneys of wild-type ($Cth^{+/+}$), heterozygous ($Cth^{+/-}$) and CTH-deficient ($Cth^{-/-}$) mice. Values plotted are mean ± SEM ($n = 4$ in sham-operated groups, $n = 8$ in I/R-injured groups). *$P < 0.05$ vs. sham-operated $Cth^{+/+}$ and sham-operated $Cth^{+/-}$; #$P < 0.05$ vs. I/R-injured $Cth^{+/+}$ and sham-operated $Cth^{+/-}$; $P < 0.05$ vs. sham-operated $Cth^{+/+}$, $Cth^{+/-}$ and $Cth^{-/-}$ mice. (**D**) Relative CTH protein levels of sham- and I/R-injured kidneys of $Cth^{+/+}$, $Cth^{+/-}$ and $Cth^{-/-}$ mice. Values plotted are mean ± SEM ($n = 4$ each). Mean CTH density of two $Cth^{+/+}$ kidneys on each gel was set to 100% and relative density was calculated for the rests. (**E**) Levels of H_2S in intact kidneys of $Cth^{+/+}$, $Cth^{+/-}$ and $Cth^{-/-}$ mice. Values plotted are mean ± SEM ($n = 11$ for $Cth^{+/+}$ and $Cth^{-/-}$, and $n = 4$ for $Cth^{+/-}$). &$P < 0.01$ vs. $Cth^{+/+}$ and $Cth^{+/-}$; §$P < 0.05$ vs. $Cth^{+/+}$ and $Cth^{-/-}$. AU, arbitrary units. n.d., not detectable.

biological samples. In addition, pharmacological CTH/CBS inhibitors have been used at suspicious high concentrations *in vivo* and *in vitro*[8], which might cause the lack of specificity in enzyme inhibition[2,9]. Therefore, transgenic animals have been recently developed to elucidate the role of H_2S in health and disease[10,11].

CTH is highly expressed in the kidney and several studies demonstrated that H_2S could exert protective effects in renal IRI. Han *et al.* found that NaHS administration to IRI mice accelerated the recovery from renal dysfunction and impaired tubular morphology, whereas the administration of DL-propargylglycine (PAG), an inhibitor of CTH, delayed it[12]. NaHS administration had beneficial effects on renal IRI[13] and gentamicin-induced acute kidney injury (AKI) in rats[14]. Tan *et al.* suggested that the beneficial effects of endogenously produced H_2S in AKI are, at least in part, mediated by toll-like receptors (TLRs)-mediated inflammatory response and apoptosis[15]. Chen *et al.* showed that exogenous H_2S reduces kidney injury from urinary-derived sepsis in rabbits, which is associated with decreased TNF-α expression in the kidneys[16]. On a conceptual level, these results are consistent with recent findings by Bos *et al.* who reported that their CTH-deficient ($Cth^{-/-}$) mice display aggravated renal IRI[10,17] when our study was underway. The authors concluded that CTH protects against renal IRI, likely by modulating oxidative stress through the production of H_2S. Autosomal-recessive cystathioninuria (OMIM 219500), which is considered as a benign biochemical anomaly, is caused by homozygous or compound heterozygous mutations in *CTH* (OMIM 607657) and has a relatively high prevalence (1 per 14,000 live births)[18] though with somewhat lower incidence in other reports[19,20]. Therefore, both the findings of Bos *et al.* and ours could have important implications for humans.

Here, we used our $Cth^{-/-}$ mice on a C57BL/6J background[11] to elucidate the function of CTH in renal IRI. We found that the systemic CTH loss does not deteriorate the immediate outcome of AKI. Instead $Cth^{-/-}$ mice displayed reduced renal damage and renal expression of inflammatory cytokines/adhesion molecules after IRI, compared to littermate control (wild-type; $Cth^{+/+}$) and heterozygous ($Cth^{+/-}$) mice.

Results

The mRNA Expression of H_2S-Generating Enzymes in the Kidney. Levels of renal *Cth* mRNA in heterozygous ($Cth^{+/-}$) mice were approximately half of those in $Cth^{+/+}$ mice (Fig. 1A). In both $Cth^{+/+}$ and $Cth^{+/-}$ mice subjected to IRI, renal *Cth* mRNA levels declined to one third of their initial expression levels (Fig. 1A). No *Cth* mRNA was detectable in the kidneys of $Cth^{-/-}$ mice (Fig. 1A). Renal mRNA levels of two other

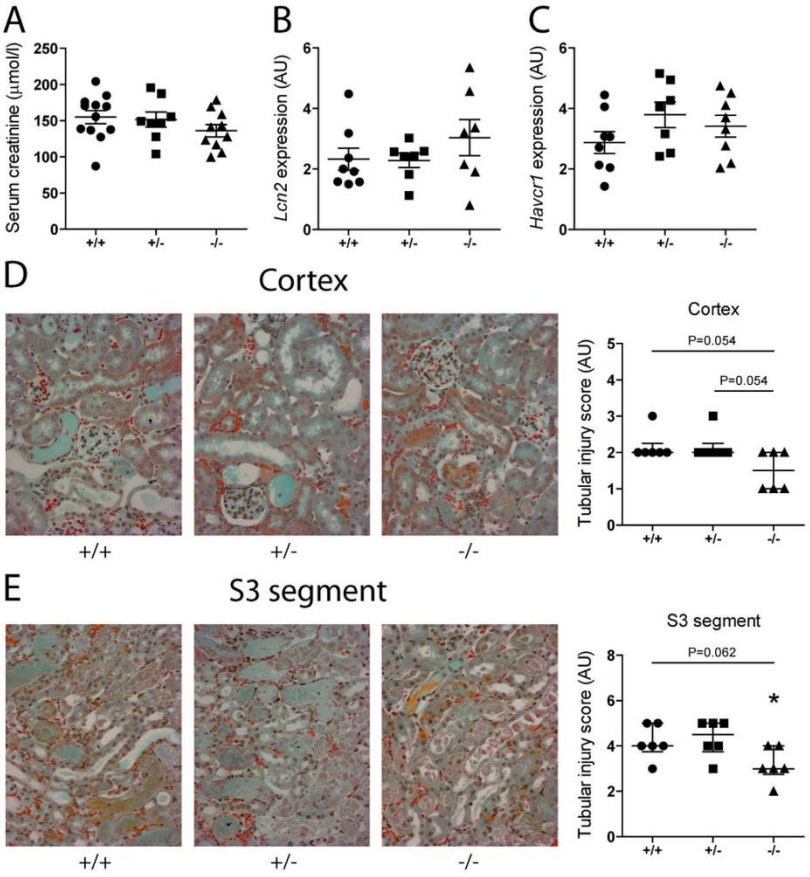

Figure 2. Renal damage after ischemia/reperfusion (I/R) injury. (**A**) Serum creatinine levels ($n = 12$, 8, and 10 for $Cth^{+/+}$, $Cth^{+/-}$, and $Cth^{-/-}$ mice, respectively) and mRNA levels of (**B**) kidney injury marker lipocalin 2 (*Lcn2*) and (**C**) hepatitis A virus cellular receptor 1 (*Havcr1*) in I/R-injured kidneys of $Cth^{+/+}$, $Cth^{+/-}$, and $Cth^{-/-}$ mice (n = 8, 7, and 8, respectively). Values plotted are mean ± SEM. (**D**) Representative cortical images of Masson's trichrome stain on sections of I/R-injured kidneys of $Cth^{+/+}$, $Cth^{+/-}$, and $Cth^{-/-}$ mice (×200). Right hand side is semi-quantification of cortical tubular injury. Values plotted are median ± interquartile range ($n = 6$ each). (**E**) Representative S3 segment images of Masson's trichrome stain on sections of I/R-injured kidneys of $Cth^{+/+}$, $Cth^{+/-}$, and $Cth^{-/-}$ mice (×200). Right hand side is semi-quantification of tubular injury. Values plotted are median ± interquartile range ($n = 6$ each). *$P < 0.05$ vs. $Cth^{+/-}$ mice. Red dots are red blood cells. AU, arbitrary units.

H_2S-producing enzymes, CBS and MPST, were not different among all three *Cth* genotypes. These levels declined markedly after IRI and the levels after IRI were also not different among *Cth* genotypes (Fig. 1B,C).

Protein Expression of CTH and Endogenous H_2S Levels in the Kidney. Levels of renal CTH protein in heterozygous ($Cth^{+/-}$) mice were ~40% of those in $Cth^{+/+}$ mice (Fig. 1D) as previously reported[11]. Similar to its mRNA level changes, renal CTH protein levels declined after IRI in both $Cth^{+/+}$ and $Cth^{+/-}$ mice and no CTH protein was detectable in the kidneys of $Cth^{-/-}$ mice (Fig. 1D). Next, we measured endogenous H_2S levels in the kidney to assess the impact of systemic CTH deletion on H_2S production. Kidneys of $Cth^{+/-}$ and $Cth^{-/-}$ mice displayed approximately 30% and 50% reduced H_2S levels, respectively, compared to those of $Cth^{+/+}$ mice (Fig. 1E).

The impact of CTH loss in Renal Damage after IRI. To get insights into (patho)physiological roles of CTH in renal IRI, we performed comparative *in vivo* studies using $Cth^{+/+}$, $Cth^{+/-}$ and $Cth^{-/-}$ mice. Twenty-four

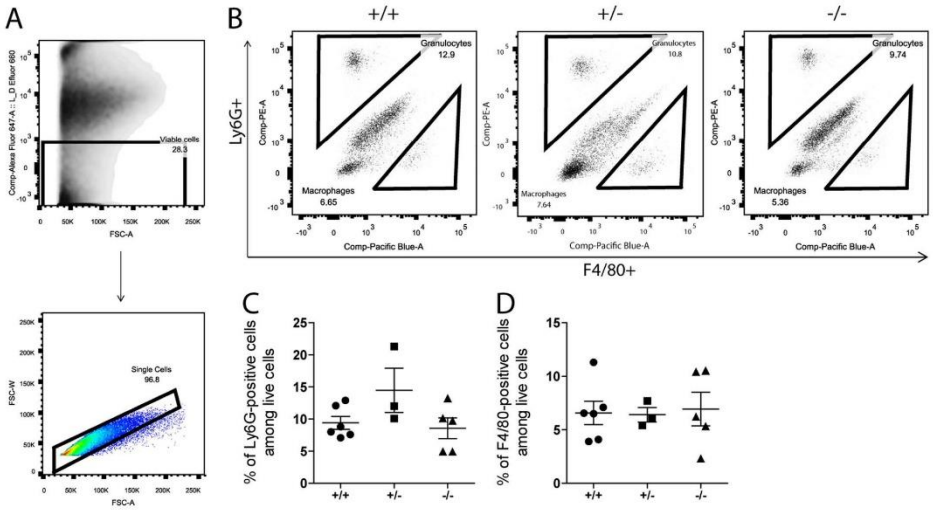

Figure 3. Flow cytometric analysis of renal granulocyte and macrophage infiltration. (**A**) Pre-gating on live cells using Fixable Viability Dye eFluor 660 and further gating on single cells. (**B**) Representative flow cytometry data of infiltrating Ly6G-positive cells (granulocytes) and F4/80-positive cells (macrophages) in I/R-injured kidneys of $Cth^{+/+}$, $Cth^{+/-}$, and $Cth^{-/-}$ mice. Quantification of infiltrating (**C**) Ly6G-positive cells and (**D**) F4/80-positive cells. Values plotted are mean ± SEM ($n = 6$, 3 and 5 for $Cth^{+/+}$, $Cth^{+/-}$, and $Cth^{-/-}$ mice, respectively).

hours after ischemia, $Cth^{-/-}$ mice showed somewhat lower serum creatinine levels (136 μmol/l average) compared to $Cth^{+/-}$ mice (152 μmol/l average) and $Cth^{+/+}$ mice (155 μmol/l average) although the differences were not statistically significant (overall ANOVA $P = 0.30$; Fig. 2A). Renal mRNA levels of two sensitive AKI markers, lipocalin 2 (*Lcn2*; also known as neutrophil gelatinase-associated lipocalin [*Ngal*]) and hepatitis A virus cellular receptor 1 (*Havcr1*; also known as kidney injury molecule 1 [*Kim1*]), were not different between $Cth^{+/+}$, $Cth^{+/-}$, and $Cth^{-/-}$ mice (Fig. 2B,C). To assess the degree of tubular damage after ischemic AKI, kidney sections were stained and examined by an experienced renal pathologist who was unaware of *Cth* genotypes. Histological analyses and semi-quantitative scoring revealed a moderate amelioration in cortical tubular damage after renal IRI in $Cth^{-/-}$ mice compared to $Cth^{+/-}$ or $Cth^{+/+}$ mice (Fig. 2D). Histological analysis of the S3 segments of the proximal tubules in the outer stripe of outer medulla, especially vulnerable loci against renal IRI, identified that $Cth^{-/-}$ mice exhibit less tubular necrosis and less occlusions of tubular lumen with cellular debris, compared to $Cth^{+/-}$ or $Cth^{+/+}$ mice (Fig. 2E). These parameters were comparable among sham-operated mice with all *Cth* genotypes (Supplementary Figure 1A–C). It is notable that all $Cth^{-/+}$, $Cth^{+/-}$ and $Cth^{-/-}$ mice that underwent surgery survived in this study.

The impact of CTH loss in Cellular Infiltration to the Kidneys after IRI. Renal IRI is known to associate with infiltration of granulocytes, monocytes/macrophages and other immune cells immediately after reperfusion, which contributes to inflammation and subsequent repair in injured kidneys[21]. Therefore, we characterized granulocytes and macrophages in renal IRI by flow cytometry. Whole kidney cell suspensions were immunolabelled for Ly6G and F4/80 as markers for granulocytes and macrophages, respectively. Among pre-gated singlet live cells (Fig. 3A), Ly6G-positive & F4/80-negative granulocytes as well as Ly6G-negative & F4/80-positive macrophages were detected (Fig. 3B). There were no significant differences in both granulocyte and macrophage populations between $Cth^{+/+}$, $Cth^{+/-}$, and $Cth^{-/-}$ kidneys at 24 h after IRI (Fig. 3C and D) or after sham surgery (Supplementary Figure 2A–C). We next performed immunohistochemistry to detect IRI-induced granulocyte infiltration (Supplementary Figure 3A)[22]. In the outer medulla after IRI, average Ly6B-positive cell numbers per view field were 12 in $Cth^{-/-}$ mice while 13 and 19 in $Cth^{+/-}$ and $Cth^{+/+}$ mice, respectively, although the differences were not statistically significant ($P = 0.501$, Supplementary Figure 3B). Furthermore, renal levels of *S100a8/a9* mRNAs for calprotectin, a heterodimeric protein that was recently found to co-localize with Ly6G in granulocytes after AKI and playing a crucial part in controlling M2 macrophage-mediated renal repair following IRI[23], were also not significantly different (Supplementary Figure 3C,D).

The impact of CTH loss in Expression of Cytokines, Chemokines, and Adhesion Molecules. Production of inflammatory molecules is maintained low in the normal kidney but is markedly increased under pathophysiological conditions such as ischemia[21]. We measured mRNA levels of several molecules involved in long-term outcome/repair after renal IRI. Renal expression of interleukin 1-beta (*Il1b*) and vascular cell adhesion

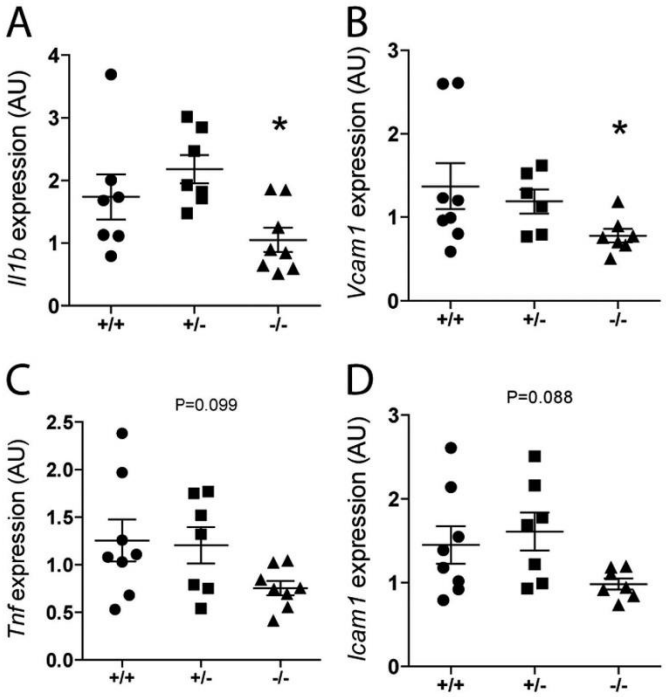

Figure 4. Renal gene expression of cytokines and adhesion molecules. Gene expression levels of (**A**) interleukin (*Il*))1-beta, (**B**) vascular cell adhesion molecule (*Vcam*)1, (**C**) tumor necrosis factor alpha (*Tnf*) and (**D**) intercellular adhesion molecule (*Icam*)1 in ischemia/reperfusion-injured kidneys of $Cth^{+/+}$, $Cth^{+/-}$, and $Cth^{-/-}$ mice. Values plotted are mean ± SEM (n = 8, 7, and 8 for $Cth^{+/+}$, $Cth^{+/-}$, and $Cth^{-/-}$ mice, respectively). *$P < 0.05$ vs. $Cth^{+/-}$ mice. AU, arbitrary units.

molecule 1 (*Vcam1*) after IRI was significantly lower in $Cth^{-/-}$ mice compared to $Cth^{+/-}$ mice (Fig. 4A,B). Also, renal expression of tumor necrosis factor-alpha (*Tnf*) and vascular cell adhesion molecule 1 (*Vcam1*) was similarly lower in $Cth^{-/-}$ mice compared to $Cth^{+/-}$ mice (overall ANOVA P = 0.099 and P = 0.088, respectively) (Fig. 4C,D). Renal expression of other important cytokines/chemokines such as interleukin 6 (*Il6*), chemokine (C-X-C motif) ligand 2 (*Cxcl2*), and chemokine (C-C motif) ligand 2 (*Ccl2*), were not altered among *Cth* genotypes (Supplementary Figure 4A–C).

The impact of CTH loss in *In Vitro* Macrophage Polarization. Although the proportion of infiltrating macrophages after IRI was not significantly different (Fig. 3D), renal mRNA expression of IL1-beta and TNF-alpha, the two major inflammatory cytokines of macrophage origin, was lower or in $Cth^{-/-}$ mice (Fig. 4A,C). We hypothesized that macrophage polarization is disturbed by the lack of CTH, and thus investigated *Tnf* induction by the lipopolysaccharide (LPS)/interferon (IFN)-gamma *in vitro* treatment of bone marrow (BM)-derived macrophages from $Cth^{+/+}$ and $Cth^{-/-}$ mice. *Cth* expression was induced while *Mpst* expression was not altered by LPS/IFN-gamma treatment in BM-derived macrophages from $Cth^{+/+}$ mice (Fig. 5A,B). In contrast, *Mpst* expression was significantly induced by the same treatment in macrophages from $Cth^{-/-}$ mice (Fig. 5B), and *Cbs* expression was not detectable in macrophages from either mice (data not shown). Under such conditions, *Tnf* expression was markedly induced by LPS/IFN-gamma treatment of both $Cth^{+/+}$ and $Cth^{-/-}$ macrophages, and the levels were significantly lower in $Cth^{-/-}$ macrophages (Fig. 5C), although the supernatant TNF-alpha concentrations of activated macrophages were comparable between $Cth^{+/+}$ and $Cth^{-/-}$ mice (Fig. 5D).

Discussion

A number of studies have demonstrated the cytoprotective effects of H_2S in myocardial, liver, brain, pulmonary, and renal IRI (reviewed by Nicholson and Calvert)[2]. Most of these studies utilized Na_2S/NaHS as exogenous H_2S donors and PAG as a non-specific CTH inhibitor. To overcome pharmacokinetic problems in H_2S donor applications and specificity issues of PAG, two research groups have independently generated mice in which *Cth* genes have been differentially deleted[10,11]. In our study, we investigated the pathophysiological roles of CTH in renal

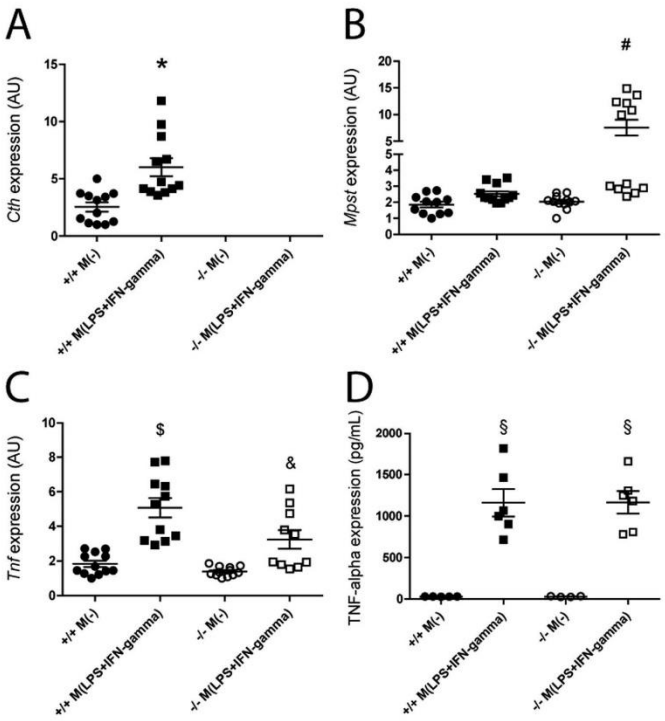

Figure 5. Gene expression of hydrogen sulfid producing enzymes and tumor necrosis alpha (Tnf) in bone marrow (BM)-derived macrophages. Gene expression levels of (**A**) cystathionine gamma-lyase (*Cth*), (**B**) 3-mercaptopyruvate sulfurtransferase (*Mpst*) and (**C**) *Tnf* in non-polarized M(−) and lipopolysaccharide (LPS) and interferon (IFN)-gamma polarized $Cth^{+/+}$ and $Cth^{-/-}$ BM-derived macrophages. Values plotted are mean ± SEM ($n = 12$ of each group, 2 independent measurement, 6 biological repetition/experiment). *$P < 0.05$ vs. $Cth^{-/+}$ M(−). #$P < 0.05$ vs. $Cth^{1/+}$ M(−), $Cth^{+/+}$ M(LPS+IFN-gamma) and $Cth^{-/-}$ M(−). $P < 0.05$ vs. $Cth^{+/+}$ M(−), $Cth^{-/-}$ M(−) and $Cth^{-/-}$ M(LPS+IFN-gamma), &$P < 0.05$ vs. $Cth^{+/+}$ M(−), $Cth^{+/+}$ M(LPS+IFN-gamma) and $Cth^{-/-}$ M(−). AU, arbitrary units. (**D**) TNF-alpha concentration in the supernatant of the cultured BM-derived macrophages. Values plotted are mean ± SEM (n = 5 and 6 for non-polarized and polarized macrophage group, respectively). §$P < 0.05$ vs. $Cth^{+/+}$ M(−) and $Cth^{-/-}$ M(−).

IRI using one of those $Cth^{-/-}$ mice and their littermate $Cth^{+/-}$ and $Cth^{+/+}$ mice as controls; all were the offspring from the mating between $Cth^{+/-}$ males and $Cth^{+/-}$ females that had been backcrossed over 10 generations onto a C57BL/6 background[11]. We found that the lack of CTH does not cause aggravated immediate renal functional impairments after IRI as assessed by serum creatinine levels (Fig. 2A) and renal expression of sensitive AKI markers, Lcn2 and Havcr1 (Fig. 2B,C). Our histological examinations rather identified a moderate amelioration in renal tubular damage in $Cth^{-/-}$ mice (Fig. 2D,E).

While our study was underway, Bos *et al.* published findings with their $Cth^{-/-}$ mice (on a mixed strain background; the sex of mice used is not indicated) investigating the role of CTH-derived H_2S in renal IRI[17]. They found that CTH deficiency aggravated kidney damage after IRI, which was associated with increased mortality[17] however, we did not observe such severe systemic damage after renal IRI. The reasons for this discrepancy are possibly multifaceted. *First*, their $Cth^{-/-}$ mice display age-dependent hypertension (15–20 mmHg higher systolic blood pressure *vs* $Cth^{+/+}$ mice only after 7 weeks of age) and sex-related hyperhomocysteinemia in which females have six times the plasma homocysteine levels (120 *vs* 20 μM) in males[10,17], both of which are caused by unknown mechanisms. Hypertension *per se* has deleterious effects on renal IRI[24,25] nevertheless, hypertension was not properly treated in their studies[17]. This affair makes it difficult to assess the impact of reduced renal H_2S production over elevated blood pressure on the outcome of IRI; fortunately, our $Cth^{-/-}$ mice display systolic normotension[11]. This fact may, at least in part, underlie differences between their and our findings. It should be noted that our $Cth^{-/-}$ males and females display similar serum levels of homocysteine (104–151 μM)[11] the reasons for this difference are yet unknown but may depend on differences in genetic backgrounds and/or nutritional conditions. *Second*, Bos *et al.* performed renal ischemia by clamping both (right and left) renal arteries for 30 min, whereas

we performed uninephrectomy by clamping the renal artery of the remnant left kidney for 20 min[17]. Despite the differences in surgical protocols, serum creatinine levels at 24 h after IRI were equivalent. But, importantly, all mice that underwent surgery survived in our study while Bos *et al.* observed 35% mortality only in $Cth^{-/-}$ mice[17]. *Third*, we used a temperature controller with heating pads to maintain a stable core temperature (which was measured continuously during surgery by a rectal probe) whereas Bos *et al.* used only heating pads and lamps[17]. It is well known that fluctuations in core body temperature contribute to variability in IRI and the way of maintaining body temperature during ischemia has a major impact on the outcome of IRI[26]. *Fourth*, their $Cth^{-/-}$ mice showed a massive (91%) reduction in renal H_2S production compared to $Cth^{+/+}$ mice[17] while our $Cth^{-/-}$ mice showed only 50% reduction (Fig. 1E). Although the methods used for H_2S measurement substantially differ between the two studies and this precludes the direct comparison, >90% reduction is surprising *per se*, considering the facts that (i) $Cth^{-/-}$ kidney still expresses CBS and MPST, (ii) (increased/activated) CBS could compensate for H_2S production when CTH is inhibited or abrogated (though we did not observe compensatory *Cbs* mRNA induction; (Fig. 1B) and (iii) renal *Cbs*/*Mpst* expression was markedly down-regulated by ischemia/reperfusion irrespective of *Cth* genotypes (Fig. 1B,C)[11,12,17,27,28]. A previous study mentioned that the reduction in CBS (rather than CTH) activity may serve as the major contributor for endogenous H_2S level reduction during renal IRI[29].

Despite such differences, we also found some agreement with previous studies by Bos *et al.*[17] and others[27,30]. First, renal expression (either gene or protein) of both CTH and CBS were suppressed after renal IRI (Fig. 1A,B). It might be noteworthy that the partial or complete loss of CTH did not cause compensatory induction (or reduced repression of expression) of CBS (or MPST) during IRI (Fig. 1B,C) at least on mRNA level. Second, both Bos *et al.* and we did not find significant differences between *Cth* genotypes in the numbers of granulocyte infiltrated into injured kidneys of IRI mice (Fig. 3C and Supplementary Figure 3A–C)[17]. We also counted the numbers of F4/80-positive macrophages infiltrated into injured kidneys of IRI mice and found that macrophages behave similar to granulocytes (Fig. 3D). In contrast, renal expression of *Tnf*, *Il1b*, *Icam1,* and *Vcam1* after IRI were lower (though overall ANOVA was just $P = 0.099$ and 0.088 for *Tnf* and *Icam1*, respectively) in $Cth^{-/-}$ mice compared to $Cth^{+/-}$ mice (Fig. 4A–D). TNF-alpha was initially discovered as a LPS-induced macrophage product[31]. It is also released during IRI and acts as a potent pro-inflammatory cytokine[32], and in line, the blockade of TNF-alpha signaling is a novel promising therapeutic target in renal IRI[33]. Although intrinsic renal cells also secret TNF-alpha upon injury, monocytes/classically activated macrophages are considered as the main source of TNF-alpha in early renal IRI[34]. We found that CTH deficiency alters *Tnf* expression in LPS/IFN-gamma-stimulated BM-derived macrophages that intrinsically differ from LPS-stimulated peritoneal macrophages[10,35]. However, the supernatant TNF-alpha concentrations did not differ between both groups, which questions the physiological relevance of this finding. Meanwhile, renal expression of other cytokines that are known to play a role in renal IRI[36] (Il6, Cxcl2, and Ccl2) were not distinguishable between *Cth* genotypes (Supplementary Figure 4A–C).

Our findings are in contrast to previous results by others who use PAG for CTH inhibition. Tripatara *et al.* found that single intraperitoneal administration of PAG (50 mg/kg, 1 h before ischemia) prevented the renal recovery from IRI (45-min ischemia/72-h reperfusion) in a rat bilateral ischemia model[37]. More recently Han *et al.* showed similar deteriorative effects of PAG in renal IRI (50 mg/kg daily (i.p.), beginning 2 days after ischemia) in mice[12]. However, PAG (5 mg/kg (i.p.), twice a day for 4 successive days) exhibited nephroprotective effects in the cisplatin model of AKI in rats[38]. Similar protective effects of PAG (50 mg/kg (i.p.) at 2 h after adriamycin injection) have been observed in adriamycin-induced nephrotoxicity in rats[39]. Whereas these kidney injury models differ, they point out that PAG treatment can have multiple effects depending on the renal injury models. Moreover, the specificity of this widely used CTH inhibitor and relatively late time points after reperfusion are a matter of concern. Our model is of particular interest because we used a genetic approach to abrogate CTH specifically and investigated acute renal post-ischemic injury after 24 h, a time point where serum creatinine levels are the highest and renal *Cth*/*Cbs* expression levels are the lowest[17].

Numerous studies have revealed cytoprotective/anti-oxidative/anti-inflammatory roles of H_2S, but some studies also have identified pro-inflammatory roles of H_2S that accelerate inflammatory responses; for example, Ang *et al.* previously reported that caerulein-induced acute pancreatic damage as well as its associated lung injury was ameliorated in $Cth^{-/-}$ mice compared to $Cth^{+/+}$[40]. It is possible that CTH-produced H_2S may act as a pro-inflammatory factor in renal IRI. In addition, further studies should also clarify the impact of high levels of cystathionine and homocysteine and low levels of taurine that are common in $Cth^{-/-}$ mice[11] on the outcome of renal IRI[41–43]. In conclusion, the systemic loss of CTH in mice caused approximately 50% reduction in renal H_2S levels but did not influence immediate outcomes of ischemic AKI; however, it reduced tubular damage moderately and suppressed the renal expression of inflammatory cytokines. Future studies should clarify the role of CTH on the long-term outcome of renal impairment in AKI.

Methods

Mice. $Cth^{+/-}$ and $Cth^{-/-}$ mice were generated and characterized earlier[11]. In this study, $Cth^{+/-}$ males and females were bred to obtain $Cth^{+/+}$, $Cth^{+/-}$, and $Cth^{-/-}$ littermates. Mice were allowed free access to standard chow and water. The mice were kept in a 12:12-h light-dark cycle. All works involving animals have been approved by the Berlin Animal Review Board in 2012 (No. G 0444/12) and conducted in accordance with the American Physiological Society standards.

Renal IRI Model. Male mice (age between 12–15 weeks) were used. Anesthesia was performed with isoflurane (2.3%) in air (350 ml/min)[44]. Each mouse was operated separately to ensure similar exposure to isoflurane (35.7 ± 2.3 min, mean ± SD)[45]. In order to keep body temperature stable at 37 °C and monitor it during surgery, a temperature controller with heating pad (TCAT-2, Physitemp Instruments) was used. Rectal body temperature was continuously monitored during surgery using a sensor-based thermistor (36.9 ± 0.4 °C at beginning of the surgery, 37.0 ± 0.4 °C after uninephrectomy, 37.1 ± 0.3 °C five minutes after clamping the left renal pedicle and

37.1 ± 0.1 °C at the end of surgery). After right-sided uninephrectomy, ischemia was induced by clipping the pedicles of the left kidney for 20 minutes with non-traumatic aneurysm clips (FE690K, Aesculap). Reperfusion was confirmed visually. After surgery, mice had free access to water and chow. We applied body-warm sterile physiological saline solutions and preemptive analgesia with tramadol (1 mg/kg) for every mouse. Sham operation was performed in a similar manner, except for clamping the renal pedicle. Mice with bleeding during surgery, with incomplete renal reperfusion, with excessive exposure of isoflurane of any reason, with significant temperature fluctuation during surgery, or with signs for infection 24 h after IRI, were immediately euthanized and were not used for further analysis. After 24 h of reperfusion, mice were sacrificed, and kidney and blood samples were collected for further analysis. The kidneys were divided into three portions. One third of the kidney was placed in optimum cutting temperature (OCT) compound for immunohistochemistry, one third was immersed in 4% phosphate-buffered saline (PBS)-buffered formalin for histology, and the rest was snap-frozen in liquid nitrogen for RNA preparation.

Quantitative Real-Time (qRT)-PCR. Total RNA from snap-frozen kidneys were isolated using RNeasy RNA isolation kit (Qiagen) according to manufacturer's instruction after homogenization with a Precellys 24 homogenizator (Peqlab). RNA concentration and quality was determined by NanoDrop-1000 spectrophotometer (Thermo Fisher Scientific). Two micrograms of RNA were transcribed to cDNA (Applied Biosystems). Quantitative analysis of target mRNA expression was performed with qRT-PCR using the relative standard curve method. TaqMan and SYBR green analysis was conducted using an Applied Biosystems 7500 Sequence Detector (Applied Biosystems). The expression levels were normalized to 18S or to beta-actin. Primer sequences are provided in Supplementary Table 1.

Western Blot. Sham and IRI-damaged kidneys were lysed with RIPA buffer (Sigma) supplemented with Complete® protease inhibitor (Roche), 1 mM phenylmethylsulfonyl fluoride (PMSF), phosphatase inhibitor cocktail 3 (Sigma) and were homogenized using a Precellys 24 homogenizator. Fifty micrograms of protein samples were separated by 12% SDS-PAGE. After wet transfer, non-specific binding sites of the nitrocellulose membrane were blocked with 5% non-fat skim milk in Tris-buffered saline containing 0.1% Tween (TBST). The membrane was then incubated with primary antibody (anti-CTH, 1:500 (ab80643) Abcam or anti-CTH carboxyl terminus rabbit polyclonal antibody that recognizes amino acids 194–398 of a rat 398-amino acid CTH protein, 1:1,000[46] and anti-beta-actin, 1:2,000 (4970) Cell Signaling). Secondary antibody was from LI-COR Biosciences (anti-rabbit, 1:5,000). Images were acquired by Odyssey infrared imaging system (LI-COR Biosciences). Beta-actin was used as a loading control. Membranes were first probed with anti-CTH antibody and detected for their signals, and then stripped for re-probing with anti-beta-actin antibody (as loading controls). Successive stripping was confirmed by the absence of signals in the stripped membranes.

TNF-alpha Measurement. TNF-alpha levels in the supernatants of macrophages (that were used for qRT-PCR analyses) were measured using the Mouse TNF alpha ELISA Ready-SET-Go!® Kit (eBioscience).

H_2S Measurement. To detect H_2S production in the kidneys, $Cth^{+/+}$ and $Cth^{-/-}$ mice were euthanized and freshly isolated kidneys were incubated in PBS containing 50 μM of a recently developed fluorescent probe (Washington State Probe-1 [WSP-1], Cayman Chemical) for H_2S[47]. After 45 min of incubation the samples were snap-frozen. Thawed samples were homogenized and centrifuged, and the supernatants were analyzed for fluorescence signals using Ex. 465 nm/Em. 525 nm using a spectrofluorometer[48]. Full spectrum was also analyzed to ensure that the measured fluorescence is indeed the product of the reaction between the probe and H_2S. Further experiments with spiking the samples with H_2S donor NaHS (10 and 50 μM) were performed to determine the accuracy of our measurements.

Serum Creatinine. Blood samples were taken from left ventricle at the time of termination. After clotting on room temperature for at least 15 min blood was centrifuged at 2,000 × g for 10 min to obtain serum. Serum creatinine was measured by external clinical laboratory (Labor 28 GmbH, Berlin).

Histology. Formalin-fixed, paraffin-embedded sections (2 μm) of kidneys were subjected to Masson's trichrome stain using standard protocols. The severity of tubular injury was assessed by a renal pathologist who is blinded to the genotype of the mice. Tubular necrosis was evaluated in a semi-quantitative manner by determining the percentage of tubules in the cortex where epithelial necrosis, loss of the brush border, cast formation, and tubular dilation was observed. A five-point scale was used: 1, normal kidney; 2: 1 to 25%; 3: 25 to 50%; 4: 50 to 75%; and 5, 75 to 100% tubular necrosis.

Immunofluorescence. Five-μm thick cryosections of IRI-injured kidneys were post-fixed in ice-cold acetone, air-dried, rehydrated and blocked with 10% normal donkey serum (Jackson ImmunoResearch) for 30 min. Then sections were incubated in a humid chamber overnight at 4 °C with rat anti-Ly6B.2 (Gr1) (1:300; MCA771G; AbD Serotec). The bound anti-Ly6B.2 antibody were visualized using Cy3-conjugated secondary antibody (1:500; Jackson ImmunoResearch) by incubating the sections for 1 h in a humid chamber at room temperature. Positive cells were counted in the outer medulla on five non-overlapping view fields at 200 × magnification and mean cell numbers were taken for analysis.

Flow cytometry. To assess granulocyte and macrophage infiltration in sham-operated in IRI-injured kidneys, single cell suspension was created with GentleMacs C-tubes (Miltenyi Biotec) in the presence of 10 mg/mL collagenase IV (Sigma) and 200 U/mL DNase I (Roche) dissolved in Hank's balanced salt solution. Dead cells were excluded from the analysis using Fixable Viability Dye eFluor 660 (eBioscience). Granulocytes and macrophages

were stained with PE-conjugated anti-Ly6G (clone: 1A8, Beckton Dickinson) and eFluor450-conjugated anti-F4/80 (clone: BM8, eBioscience) antibodies, respectively. Samples were analyzed on FACSCanto II flow cytometer (Becton Dickinson). Data analysis was conducted by FlowJo (TreeStar) software.

Preparation and activation of BM-derived macrophages. Cells were isolated from the femur and tibia of freshly euthanized $Cth^{+/+}$ and $Cth^{-/-}$ mice, by flushing with approximately 10 ml of activation media (RPMI1640 containing L-glutamine (Gibco), 10% (v/v) fetal calf serum (FCS), 10 mM HEPES, 50 μM beta-mercaptoethanol, 1% (v/v) penicillin/streptomycin (P/S), without colony stimulating factor (CSF)-1). Cells were then pelleted, and resuspended in monocyte differentiation media (DMEM (Gibco), 10% (v/v) FCS, 5% (v/v) adult horse serum (Cell Concepts), 1:100 non-essential amino acids (Sigma), 50 μM beta-mercaptoethanol (Sigma), with 20% (v/v) L929 conditioned media containing CSF-1 Gibco® RPMI. Conditioned media containing CSF-1 was generated by collecting the media from L929 cells (ATCC) cultured for 14 days in DMEM containing 10% (v/v) FCS, 1:100 non-essential amino acids, 10 mM HEPES and 1% (v/v) P/S. For macrophage differentiation, 10^7 bone-marrow cells were cultivated in 50 ml of differentiation media for 7 days in sealed, hydrophobic Teflon® bags (FT FEP 100 C (DuPont), American Durafilm) at 37 °C and 10% CO_2. The yield of BM-derived M(−) macrophages (also known as M0) from one bag was consistently approximately $7–10 \times 10^7$cells with a purity of >95% (determined as F4/80+ CD11b+ cells by flow cytometry). For activation of M(−) into M(LPS+IFN-gamma) (also known as M1), BM-derived M(−) were harvested from Teflon bags, pelleted and resuspended into activation media containing LPS (100 ng/ml) and recombinant mouse IFN-gamma (20 ng/ml). For qRT-PCR analysis, 2×10^6 BMD-derived M(−) and BM-derived M(LPS+IFNgamma) were plated per well of 6-well plates. In all cases, the M(−) macrophages were first allowed to rest and adhere for 2 h. Then cells were activated for 24 h at 37 °C and 5% CO_2.

Statistics. Statistical analysis was performed using GraphPad 5.04 (GraphPad Software) and SPSS 13.0 (SPSS) softwares. Normality of the data was evaluated by Kolmogorov-Smirnov test. To test the presence of an outlier, Grubbs' test was used. Study groups were analyzed by one-way ANOVA using Tukey's post-hoc test or by Games-Howell post-hoc test if homogeneity of variances was violated, with the exception of tubular necrosis data. Those were analyzed using Kruskal-Wallis test and Mann Whitney U-test. Data are presented as mean ± SEM, or median and interquartile range in case of tubular necrosis data. P values < 0.05 were considered as statistically significant.

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Author Contributions

L.M. designed the experiments, performed Western blots, analyzed data and drafted the manuscript. I.A.Sz. contributed substantially to conception of the study and revised the manuscript critically for important intellectual content. M.R.F. performed H_2S measurements and revised the manuscript. M.K. performed genotyping of the mice and revised the manuscript. A.B. performed the flow-cytometry experiments. J.-K. P. performed the histological evaluation of kidneys. L.P. performed all experiments with bone marrow-derived macrophages. G.N. performed ischemia-reperfusion surgery and collected blood and tissues. S.K. contributed to the drafting of the manuscript and revised the manuscript. J.A. performed the histological evaluation of granulocyte influx in kidneys. I.I. supplied the animals and revised the manuscript critically for important intellectual content. D.N.M. designed experiments with macrophages, supervised the experimental work, revised the manuscript critically for important intellectual content. M.G. supervised the experimental work, contributed to the drafting of the manuscript and revised the manuscript critically for important intellectual content. Final approval of the version to be published was obtained. All authors agree to be accountable for all aspects of the work in ensuring that questions related to the accuracy or integrity of any part of the work are appropriately investigated and resolved.

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3. Role of TRPC6 in kidney damage after acute ischemic kidney injury

Zheng Z, Tsvetkov D, Bartolomaeus TUP, Erdogan C, Krügel U, Schleifenbaum J, Schaefer M, Nürnberg B, Chai X, Ludwig FA, N'diaye G, Köhler MB, Wu K, Gollasch M, **Markó L**. Sci Rep. 2022;12(1):3038.

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The kidney can be the primary source of its disease (primary nephrological clinical entities), but the pathogenic processes often originate outside the kidney (secondary kidney diseases).[4] Dysfunction of ion channels (membrane proteins that form pores and thereby allow ions to pass through the cell membrane) or their interacting proteins, either of genetic or acquired reasons, can lead to primary renal clinical disease. As such, in 2005, the familiar form of focal segmental glomerular sclerosis (FSGS) was described as a gain-of-function mutation in the canonical transient receptor potential (TRPC) 6-channel gene.[34] TRPC channels belong to the TRP superfamily[93] and are the most intensely studied family of channels in glomerular cells.[94] In humans, they have 6 subtypes (designated as TRPC1-7, whereas TRPC2 is a pseudogene). TRPC6, similarly to the other TRPC channels, has a broad expression profile in the kidney. In mammals, it is expressed in mesangial cells,[95] podocytes,[35] vascular smooth muscle cells,[96], and in principal cells of the collecting duct and scattered in some renal cortex cells.[97] The TRPC6 channel can be activated by different stimuli, including stretch activation,[98] redox mechanism[99], or diacylglycerol, generated by the activation of G protein-coupled receptors.[100] Activation of the TRPC6 channel leads mainly to Ca^{2+} influx resulting in changes in cell motility or to the secretion of cellular vesicles[96], but TRPC6 channels are often part of a larger molecular signaling complex.[101] Because mutations of the TRPC6 channels can lead to glomerulopathy leading to excessive fibrosis, and the activation of the channels involves mechanisms which are also playing a significant role in renal damage signaling pathways, it became fast a target molecule for renoprotection.

Studies investigating renal fibrosis using the obstructive nephropathy model reported reductions in tubulointerstitial fibrosis in TRPC6 knockout mice[40,102] and wild-type mice treated with TRPC channel inhibitors.[39,103] Knocking out the TRPC6 channel has been additionally shown to reduce glomerular damage in several acquired glomerular disease models. Still, this

strategy was less effective in alleviating diabetic nephropathy in mouse and rat models.[104] However, if modulation of TRPC6 channels can be used as a nephroprotective approach in acute renal diseases such as AKI has not been considered, although reactive oxygen species produced during ischemic injury could possibly modify the activation of TRPC6 channels. Additionally, the TRPC6 channel has been shown to play a role in transendothelial immune cell migration and C-X-C motif chemokine receptor 2-mediated neutrophil chemotaxis. These mechanisms are crucial in the early phase of AKI.[105,106] We used wild-type and TRPC6 knockout ($TRPC6^{-/-}$) mice[96] and applied specific, orally bioactive TRPC6 inhibitors (SH045 and BI-749327) to explore the potential nephroprotective effect of such an approach during the early stage of AKI.

At baseline, $TRPC6^{-/-}$ mice had comparable blood parameters to wild-type mice (except elevated but physiological Na^+ and ionized Ca^{2+} concentrations). After a 20-minute ischemia and 24 hours reperfusion injury (IRI), wild-type and $TRPC6^{-/-}$ mice developed similarly high serum creatinine, blood urea nitrogen, and potassium concentrations. Renal histopathology, as well as the extent of renal neutrophil granulocyte infiltration and renal mRNA expression of various inflammatory cytokines (such as tumor necrosis factor-alpha (TNFα) or interleukin (IL) 6) or adhesion molecules (such as vascular cell adhesion molecule 1 or intercellular adhesion molecule 1) were also comparable between the WT and $TRPC6^{-/-}$ mice that underwent IRI. Since the $TRPC6^{-/-}$ mice we used (with a mixed, 129Sv:C57BL/6J genetic background) were established as a homozygous lineage, we could not use real littermates as control animals; therefore, mice of C57BL/6J genetic background were used as control wild-type animals. To overcome this potential confounding factor, we further investigated the effect of 2 specific TRPC6 inhibitors, namely SH045 and BI-749327. SH045 was applied intravenously (through the tail vein) 30 minutes before ischemia induction, and BI-749327, since its orally bioavailable, via oral gavage 60 min before induction of renal ischemia. Additionally, we randomly assigned mice to two ischemic times: a milder one of 17.5 minutes and another with 20 minutes of ischemia, which induces more severe damage. High concentrations of the injected SH045 were detected by high-performance liquid chromatography-tandem mass spectrometry in the renal tissue of mice 30 minutes as well as 24 hours after IRI. Still, similarly to the findings of the experiments with the $TRPC6^{-/-}$ mice, we could not detect any effect of the applied inhibitors on the immediate outcome of renal IRI. Using isolated kidneys and perfused with SH045 or BI-749327, we also could not see any impact of TRPC6 inhibition on the renal arterial myogenic tone.

Whereas we could not detect any effect on the immediate outcome of AKI by lacking or inhibiting TRPC6 channels, a recent study using pharmacological inhibition of TRPC6 in mice

(though using different TRPC6 channel blockers) demonstrated reduced astrocytic apoptosis as well as cytotoxic and inflammatory responses in cerebral IRI model.[107] More intriguingly, as our study was on its way, Hou et al. published renoprotective effect of lacking TRPC6 in a renal IRI model.[108] They observed that lacking TRPC6 leads to ameliorated IRI-induced renal damage by increasing the level of autophagy and restraining mitochondria-mediated apoptosis. The discrepancy between their and our findings has probably multiple reasons. For example, crucial experimental circumstances, such as the genetic background of the mice or the renal IRI protocol, were different between the two studies.

In summary, despite a multimodal experimental approach, we could not provide evidence that inhibition of TRPC6 channels would be beneficial to ameliorate outcomes in the early phase of AKI. However, others could detect beneficial effects by either inhibiting or lacking TRPC6 in a cerebral IRI or even in a different renal IRI model, respectively. These results warrant further research and make TRPC6 a potential druggable target for treating acute renal injury.

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Role of TRPC6 in kidney damage after acute ischemic kidney injury

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Transient receptor potential channel subfamily C, member 6 (TRPC6), a non-selective cation channel that controls influx of Ca^{2+} and other monovalent cations into cells, is widely expressed in the kidney. *TRPC6* gene variations have been linked to chronic kidney disease but its role in acute kidney injury (AKI) is unknown. Here we aimed to investigate the putative role of TRPC6 channels in AKI. We used *Trpc6*$^{-/-}$ mice and pharmacological blockade (SH045 and BI-749327), to evaluate short-term AKI outcomes. Here, we demonstrate that neither *Trpc6* deficiency nor pharmacological inhibition of TRPC6 influences the short-term outcomes of AKI. Serum markers, renal expression of epithelial damage markers, tubular injury, and renal inflammatory response assessed by the histological analysis were similar in wild-type mice compared to *Trpc6*$^{-/-}$ mice as well as in vehicle-treated versus SH045- or BI-749327-treated mice. In addition, we also found no effect of TRPC6 modulation on renal arterial myogenic tone by using blockers to perfuse isolated kidneys. Therefore, we conclude that TRPC6 does not play a role in the acute phase of AKI. Our results may have clinical implications for safety and health of humans with *TRPC6* gene variations, with respect to mutated TRPC6 channels in the response of the kidney to acute ischemic stimuli.

Transient receptor potential (TRP) channels are a group of ion channels located mostly on the plasma membrane of numerous cell types with a relatively large non-selective permeability to cations[1,2]. Mammalian TRP channel family comprises 28 members, which share some structural similarity with each other[2,3]. Transient receptor potential canonical or classical 6 (TRPC6) are non-selective Ca^{2+} permeable cation channels expressed in renal tissue including glomerular podocytes, mesangial cells, endothelial cells, tubulointerstitial vascular and epithelial cells, as well as in renal blood vessels[4]. Ca^{2+} influx through TRPC6 maintains the integrity of glomerular filtration barrier by interacting with nephrin, podocin, CD2-associated protein, and α-actinin-4 directly or indirectly[5]. Mutations in *TRPC6* lead to familial forms of focal segmental glomerulosclerosis (FSGS) and to end stage kidney disease[6,7]. Of note, TRPC6 dysregulation is also linked to progression of acquired forms of proteinuric kidney disease[8,9]. As a result of the TRPC6 activation, intracellular Ca^{2+} concentration in the podocyte increases and ultimately causes programmed cell death leading to progressive kidney failure. Recent evidence strongly suggests that TRPC6 also contributes to renal fibrosis and immune cell infiltration in the unilateral ureteral obstruction mouse model of progressive renal interstitial fibrosis[9,10].

Initiation of renal fibrosis is often caused by acute kidney injury (AKI), an increasingly common complication occurring in critically ill patients with high morbidity and mortality[11]. The outcome of AKI has been

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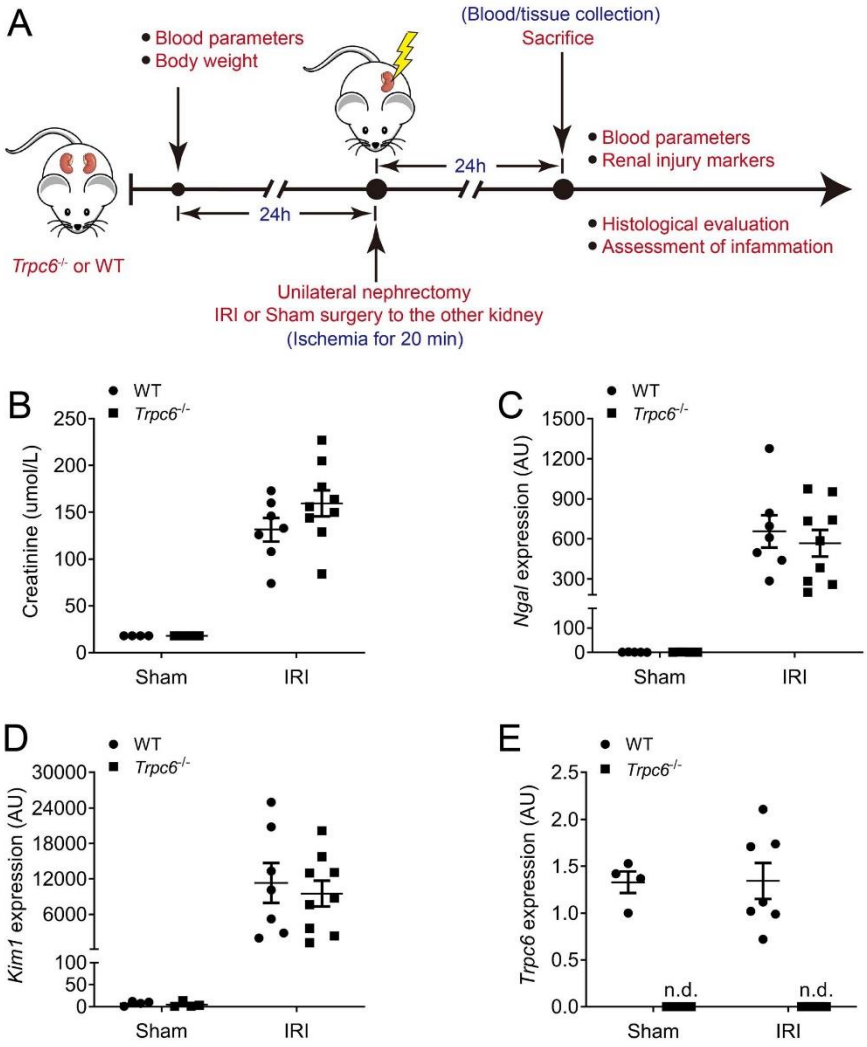

Figure 1. Effect of *Trpc6* deficiency on IRI-induced acute kidney injury (AKI). (**A**) Experimental design of ischemia reperfusion-induced AKI. (**B**) Serum creatinine levels in the experimental groups (Sham WT and *Trpc6*$^{-/-}$ n = 4 each, IRI WT n = 7, and *Trpc6*$^{-/-}$ n = 9, respectively). (**C**) Renal mRNA levels of kidney injury marker neutrophil gelatinase-associated lipocalin (*Ngal*), (**D**) kidney injury molecule 1 (*Kim1*) and (**E**) *Trpc6* (Sham WT and *Trpc6*$^{-/-}$ n = 4 each, IRI WT n = 7, and *Trpc6*$^{-/-}$ n = 9, respectively). Please note that at baseline all serum creatinine levels were below the measurement limit (18 µmol/L). Two-way ANOVA followed by Sidak's multiple comparisons post hoc test. *AU* arbitrary units, *n.d.* not detected.

a stronger with 20 min) (Fig. 5A). Renal concentrations of SH045 in both 30 min and 24 h after intravenous injection were similar between sham and IRI groups (Fig. 5B). Consistent with AKI data from *Trpc6*$^{-/-}$ and WT mice, pharmacological blockade of TRPC6 had no effects on serum creatinine levels after 17.5 min or 20 min IRI-induced AKI in comparison to vehicle injected WT IRI mice ($P = 0.23$ and $P = 0.76$, respectively, Fig. 5C). In addition, renal mRNA expression of *Ngal* and *Kim1* was similar in SH045-treated compared to vehicle-treated kidneys after IRI (Fig. 5D,E). Furthermore, SH045 did not affect blood parameters such as sodium, potassium, chloride, ionized calcium, carbon dioxide, glucose, urea nitrogen, hematocrit, hemoglobin, and the anion gap

primarily linked to the severity of tubular damage. In fact, most theories consider renal tubular cells as the main culprit in AKI. Ischemia/reperfusion injury (IRI)-induced epithelial damage—particularly in the outer medulla-, tubular obstruction, Ca^{2+} overload, loss of cytoskeletal integrity, and loss of cell–matrix adhesion are main consequences of ischemia and initiators of subsequent fibrosis[12,13]. Recent data show that in vivo TRPC6 inhibition by BI-749327 ameliorates renal fibrosis in the unilateral obstructive nephropathy (UUO) model[14], where the primary feature is tubular injury as a result of obstructed urine flow. Ischemia is frequently involved in AKI and correlates with oxidative stress and inflammation[15,16]. Reactive oxygen species (ROS) produced by NADPH oxidases (NOX) contribute to activation of TRPC6 channels[17,18]. Moreover, TRPC6 is emerging as a functional element to control calcium currents in immune cells, thereby regulating transendothelial migration, chemotaxis, phagocytosis, and cytokine release[19,20]. In a recent study, Shen et al. found that silencing TRPC6 could prevent necroptosis of renal tubular epithelial cells upon IRI[21]. These findings indicate that inhibition of TRPC6 may protect the kidney from IRI making it a promising target to ameliorate AKI. This is of special interest and of potential clinical relevance as novel chemical substances selectively acting on TRPC6 have been recently developed for in vivo experiments. In particular, the diterpene (+)-larixol and its derivative SH045, as well as BI-749327 which is another known TRPC6 blocker, have been developed for selective inhibition of TRPC6 and translational treatment of TRPC6 channelopathies[14,22,23].

To the best of our knowledge, no studies investigated the potential role of TRPC6 channels in AKI. Therefore, we tested the hypothesis that TRPC6 inhibition is renoprotective in AKI. We induced AKI by ischemia reperfusion injury in wild-type (WT) and *Trpc6*$^{-/-}$ mice. Furthermore, we used pharmacological blockers (SH045 and BI-749327) in vivo and ex vivo to verify findings from the genetical model and to evaluate effects of TRPC6 inhibition in IRI-induced AKI and intrarenal regulation of blood flow. Serum markers, renal damage marker expression, histological analysis of renal tissue damage and cellular infiltration were performed. We conclude that neither lacking nor pharmacological inhibition of TRPC6 ameliorates short-term outcomes of AKI.

Results

The impact of *Trpc6* deficiency on renal damage after renal IRI. To examine a possible role of *Trpc6* deficiency in AKI, we performed comparative in vivo studies using *Trpc6*$^{-/-}$ and WT mice (Fig. 1A). Twenty-four hours after IRI WT mice showed similar serum creatinine level compared to *Trpc6*$^{-/-}$ mice ($P = 0.18$; Fig. 1B). Renal expression of tubular damage marker neutrophil gelatinase-associated lipocalin (*Ngal*) and kidney injury molecule 1 (*Kim1*) showed no difference between WT and *Trpc6*$^{-/-}$ mice (Fig. 1C,D). Additionally, *Trpc6* expression in kidneys after IRI was not altered compared to the sham kidneys (Fig. 1E). At baseline *Trpc6*$^{-/-}$ mice had similar blood parameters compared to WT mice (except higher but still normal concentrations of sodium and ionized Ca^{2+} (Supplementary Table S2A). After IRI, WT and *Trpc6* deficient mice developed similar hyperkalaemia and similar high serum levels of creatinine and urea nitrogen (Supplementary Table S2B). In contrast, concentrations of sodium and chloride were lower in *Trpc6*$^{-/-}$ mice (Supplementary Table S2B).

The impact of *Trpc6* deficiency on renal histopathology after IRI. To assess the degree of tubular damage in ischemic AKI kidney sections were analyzed by Periodic Acid-Schiff (PAS) staining (Fig. 2A). IRI induced severe tubular damage and necrosis such as tubular epithelial swelling, loss of brush border, luminal dilatation with simplification of the epithelium, patchy loss of tubular epithelial cells with resultant gaps and exposure of denuded basement membrane, as well as obliterated tubular hyaline and/or granular casts (Fig. 2A). Analysis of the kidney sections revealed no differences in tubular injury score and tubular necrosis score between *Trpc6*$^{-/-}$ and WT mice (Fig. 2B,C).

The impact of *Trpc6* deficiency on cellular infiltration and on the expression of calcium-binding proteins in the kidneys after IRI. Neutrophils infiltration after IRI-induced AKI contributes to inflammation and subsequent repair of injured kidneys[24,25]. Here, we determined neutrophils infiltration in renal IRI using immunofluorescence staining. Kidney sections were immunolabelled with the neutrophil marker Ly6B.2[26]. As shown in Fig. 3A, excessive Ly6B.2-positive neutrophils in renal interstitium were observed in mice underwent IRI (Fig. 3A). *Trpc6*$^{-/-}$ mice showed similar amount of infiltratating Ly6B.2-positive cells after IRI as WT mice (Fig. 3B). Similarly, renal expression of neutrophil markers S100 calcium-binding protein A8 (*S100a8*) and S100 calcium-binding protein A9 (*S100a9*) were not different in IRI-damaged renal tissues of *Trpc6*$^{-/-}$ and WT mice (Fig. 3C,D).

Expression of inflammatory markers in WT and *Trpc6*$^{-/-}$ kidneys. Expression of inflammatory molecules is low in the normal kidney but is markedly increased under pathophysiological conditions such as IRI-induced AKI[27]. By using qPCR, we examined the mRNA expression of cell adhesion molecules and inflammatory markers involved in renal IRI. IRI led to increased mRNA expression of all determined markers (Fig. 4A–F), however *Trpc6*$^{-/-}$ IRI kidneys displayed similar expression of interleukin 6 (*Il6*), tumor necrosis factor-alpha (*Tnf-a*), intercellular adhesion molecule 1 (*Icam1*), vascular cell adhesion molecule 1 (*Vcam1*) in comparison to WT IRI kidneys (Fig. 4A–D). In addition, renal expression of chemokines such as chemokine (C–C motif) ligand 2 (*Ccl2*) and chemokine (C–C motif) ligand 2 receptor (*Ccr2*), were similar between *Trpc6*$^{-/-}$ and WT mice (Fig. 4E,F).

The impact of Trpc6 pharmacological blockade on renal damage after renal IRI. To circumvent possible bias due to the genetical approach and to examine effects of pharmacological blockade of Trpc6 on renal damage, we used SH045 (a recently developed drug with high affinity and strong subtype selectivity toward TRPC6) in vivo in the ischemic AKI mouse model using two different ischemia time (milder with 17.5 min and

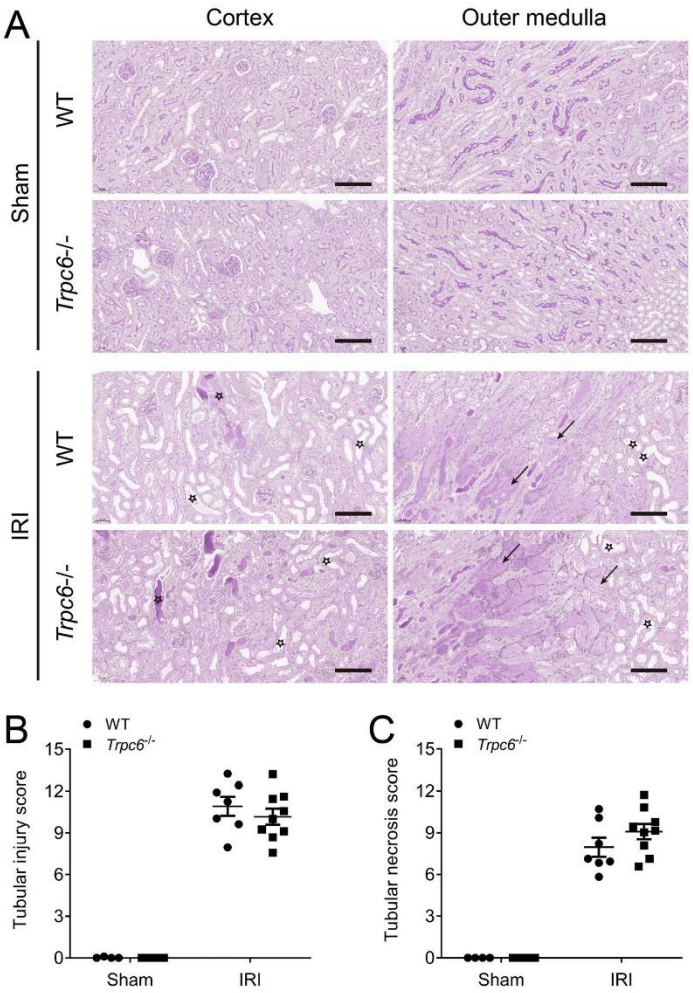

Figure 2. Effect of *Trpc6* deficiency on kidney histopathology after renal IRI–induced AKI. (**A**) Representative cortical or outer medullary images of IRI-injured kidneys isolated from *Trpc6*$^{-/-}$ and WT mice (magnification: 200×). Kidneys sections were stained with periodic acid-Schiff staining (PAS). Arrows indicate tubular necrosis. Stars indicate tubular injury. Scale bars are 100 µm. (**B**) Semi-quantification of cortical tubular injury. (**C**) Semi-quantification of outer medullary tubular necrosis. Data expressed as means ± SEM (Sham WT and *Trpc6*$^{-/-}$ n = 4 each, IRI WT n = 7, and *Trpc6*$^{-/-}$ n = 9, respectively). Two-way ANOVA followed by Sidak's multiple comparisons post hoc test.

after IRI-induced AKI (Supplementary Table S3A–F). To further confirm the results of SH045, we used another TRPC6 blocker, BI-749327. In IRI-induced AKI mice, renal function parameters such as serum creatinine and renal expression of renal damage markers (*Ngal* and *Kim1*) were similar between vehicle- and BI-749327-treated mice (Supplementary Fig. S1A–D). Besides, no difference in other blood parameters was found between vehicle- and BI-749327-treated AKI mice (Supplementary Table S4A,B).

The impact of Trpc6 pharmacological blockade on renal histopathology after IRI. Histological analyses and semi-quantitative scoring revealed no statistically significant differences in cortical tubular damage

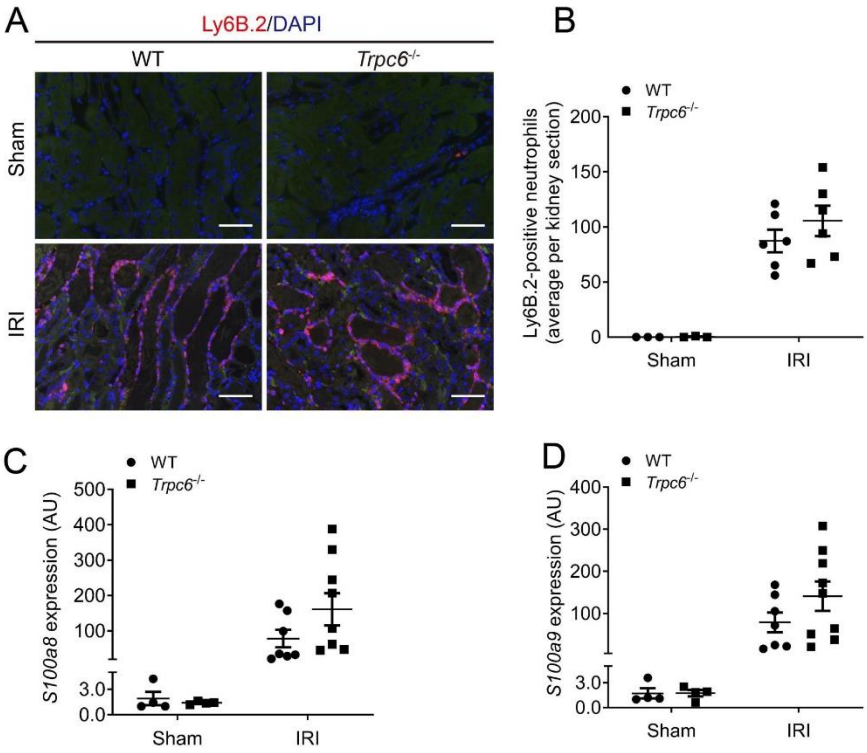

Figure 3. Effect of *Trpc6* deficiency on renal neutrophils infiltration and inflammatory markers in IRI–induced AKI. (**A**) Representative outer medullar images of IRI-injured kidneys. Kidneys were stained with Ly6B.2 (magnification: 400×). Scale bars are 100 µm. (**B**) The semi-quantification renal neutrophils infiltration (Sham WT and *Trpc6*$^{-/-}$ n = 3 each, IRI WT n = 6, and *Trpc6*$^{-/-}$ n = 6, respectively). (**C**) Renal mRNA levels of S100 calcium-binding protein A8 (*S100a8*). (**D**) Renal mRNA levels of S100 calcium-binding protein A9 (*S100a9*) (Sham WT and *Trpc6*$^{-/-}$ n = 4 each, IRI WT n = 7, and *Trpc6*$^{-/-}$ n = 9, respectively). Data expressed as means ± SEM. Two-way ANOVA followed by Sidak's multiple comparisons post hoc test. *AU* arbitrary units.

after renal 17.5 min- or 20 min-IRI in SH045 compared to vehicle-treated mice (Fig. 6A,B). Moreover, epithelial cells in the outer stripe of outer medulla, especially susceptible to ischemic injury, exhibited comparable level of tubular necrosis between SH045 treated and control mice after renal 17.5 min- or 20 min-IRI (Fig. 6A,C). Similarly, BI-749327 also did not influence tubular damage and necrosis in IRI (Supplementary Fig. S2A–C).

The impact of Trpc6 pharmacological blockade on cellular infiltration and calcium-binding proteins on the kidneys after IRI. Consistent with the results in AKI-induced *Trpc6*$^{-/-}$ mice, SH045 treatment had no effect on renal infiltration of Ly6B.2-positive granulocytes after 17.5 min- or 20 min-IRI-induced AKI in comparison to vehicle-treated mice (Fig. 7A,B). In agreement with that, renal expression of *S100a8* and *S100a9* after 17.5 min- or 20 min-IRI is similar in mice treated with SH045 versus vehicle (Fig. 7C,D). Furthermore, similar results were also obtained in mice treated with BI-749327 compared to vehicle-treated mice (Supplementary Fig. S3G,H).

The impact of Trpc6 pharmacological blockade on expression of renal cytokines and chemokine after IRI. SH045-treated mice underwent 17.5 or 20 min IRI-induced AKI showed similar renal expression of *Il6*, *Tnf-α*, *Icam1*, *Vcam1*, *Ccl2*, and *Ccr2* in comparison to vehicle-treated mice with the same IRI (Fig. 8A–F). In addition, the mRNA levels of these inflammatory markers in BI-749327-treated AKI mice were equivalent to vehicle-treated AKI mice (Supplementary Fig. S3A–F).

Impact of pharmacological modulation of Trpc6 on renal microcirculation. The renal microcirculation is emerging as a key player in AKI[28]. To explore whether modulation of TRPC6 has impact on renal microcirculation, we evaluated myogenic tone in the mouse renal circulation using the TRPC6 blockers SH045

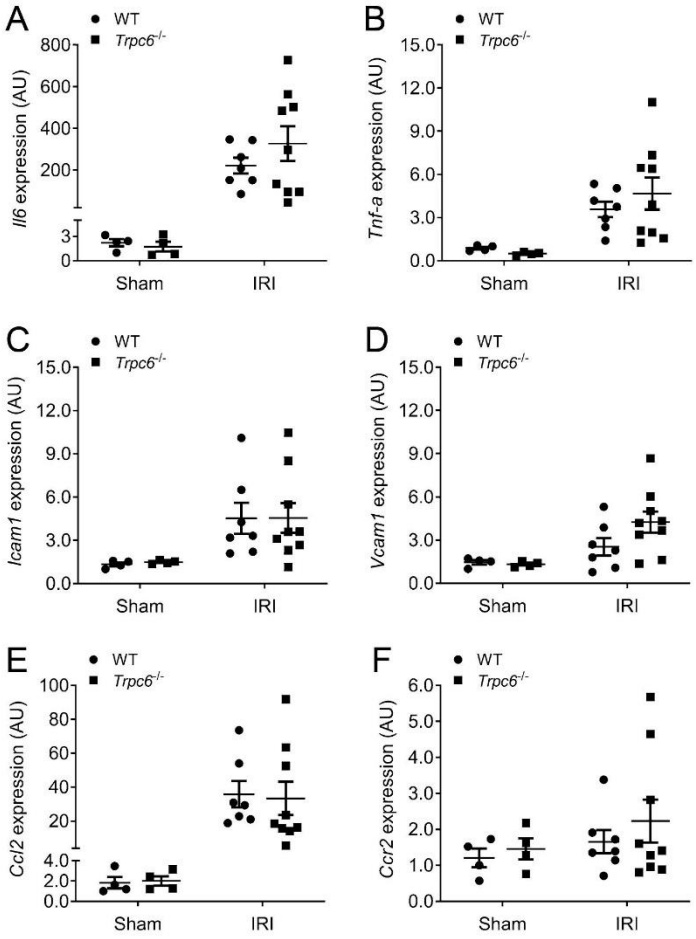

Figure 4. Effect of *Trpc6* deficiency on gene expression of pro-inflammatory cytokines and chemokines after renal IRI. (**A**) Renal mRNA levels of interleukin 6 (*Il6*) and (**B**) tumor necrosis factor-α (*Tnf-α*), (**C**) intercellular adhesion molecule 1 (*Icam1*) and (**D**) vascular cell adhesion protein 1 (*Vcam1*), (**E**) C–C motif chemokine 2 (*Ccl2*) and (**F**) C–C motif chemokine receptor 2 (*Ccr2*) (Sham WT and $Trpc6^{-/-}$ n = 4 each, IRI WT n = 7, and $Trpc6^{-/-}$ n = 9, respectively). Data expressed as means ± SEM. Two-way ANOVA followed by Sidak's multiple comparisons post hoc test. *AU* arbitrary units.

and BI-749327, and the TRPC6 activator hyperforin[29]. Isolated kidneys perfused with SH045 or BI-749327 developed a similar decrease of perfusion pressure compared to control kidneys (Supplementary Fig. S4A–C,E). In addition, activation of TRPC6 using hyperforin resulted in similar spontaneous decrease of perfusion pressure compared to control kidneys (Supplementary Fig. S4A,D,E). Of note, there was also no difference in Angiotensin (Ang) II induced vasoconstrictions between kidneys perfused with SH045, BI-749327, or hyperforin versus control kidneys (Supplementary Fig. S4F). Together, these results indicate no effect of TRPC6 modulation on renal arterial myogenic tone.

Discussion

In our previous study, we have shown that TRPC6 contributes to renal fibrosis and immune cell infiltration in a murine UUO model using $Trpc6^{-/-}$ mice[9]. Renal IRI is a common cause of CKD. IRI is associated with calcium (Ca^{2+}) overload, ROS production, and immune responses which has been reported as crucial factors of tubular

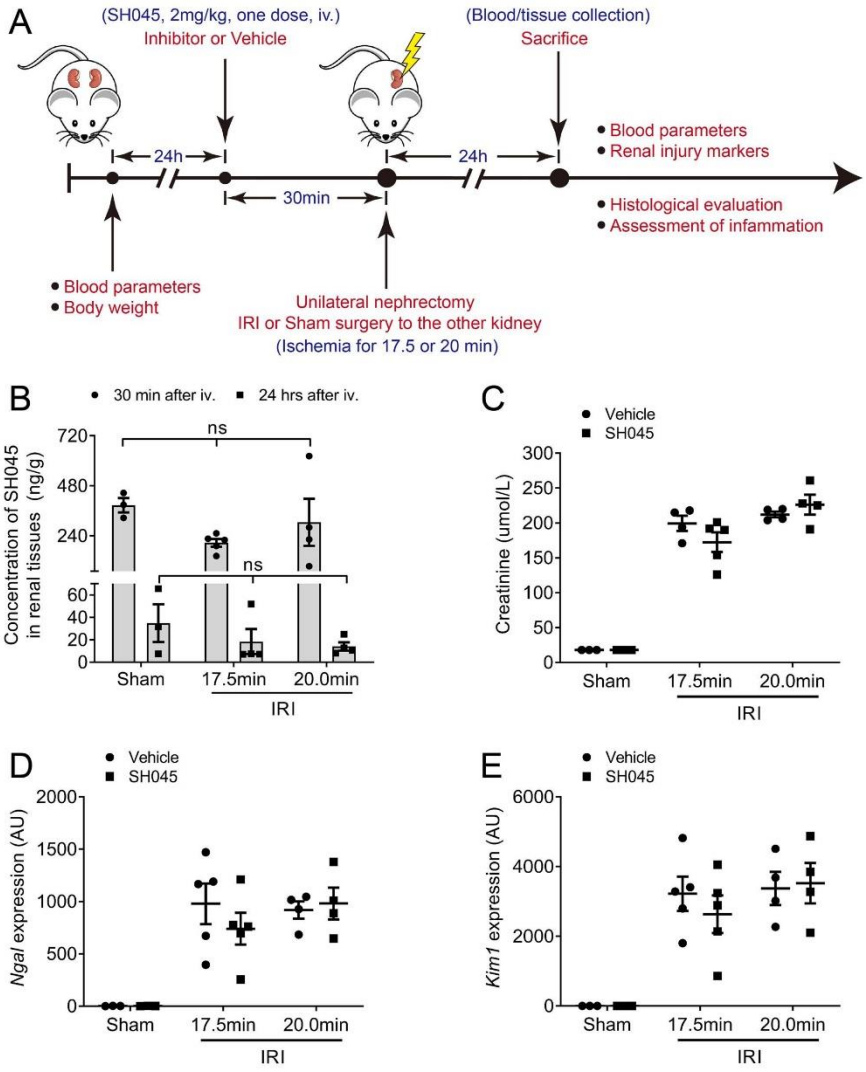

Figure 5. Effect of the TRPC6 inhibitor SH045 on IRI–induced acute kidney injury (AKI). (**A**) Experimental design of IRI–induced AKI. (**B**) Concentrations of SH045 in kidney tissue 30 min and 24 h after intravenous injection (Sham n = 3 each, 17.5 min-IRI in vehicle and SH045 n = 5 each, 20 min-IRI in vehicle and SH045 n = 4 each, respectively). (**C**) Serum creatinine levels (Sham n = 3 each, 17.5 min-IRI in vehicle and SH045 n = 5 each, 20 min-IRI in vehicle and SH045 n = 4 each, respectively). (**D**) Renal mRNA levels of kidney injury marker neutrophil gelatinase-associated lipocalin (*Ngal*) and (**E**) kidney injury molecule 1 (*Kim1*) (Sham n = 3 each, 17.5 min-IRI in vehicle and SH045 n = 5 each, 20 min-IRI in vehicle and SH045 n = 4 each, respectively). Please note that at baseline all serum creatinine levels were below the measurement limit (18 µmol/L). Two-way ANOVA followed by Sidak's multiple comparisons post hoc test. *AU* arbitrary units. Data expressed as means ± SEM.

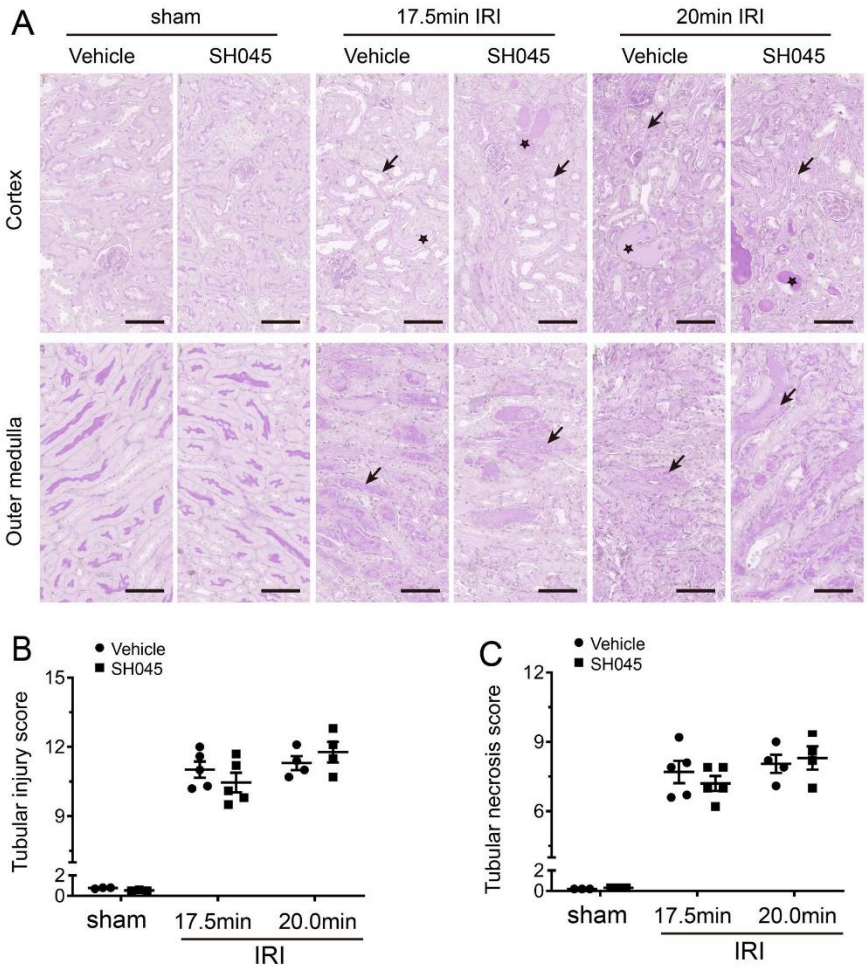

Figure 6. SH045 effects on kidney histopathology after renal ischemia reperfusion–induced AKI. (**A**) Representative cortical or outer medullary images of IRI-injured kidneys isolated from mice injected with vehicle or Trpc6 blocker SH045 (magnification: 400×). Kidneys sections/slices were stained with Periodic Acid-Schiff staining (PAS). Arrows indicate tubular injury. Stars indicate tubular casts. Scale bars are 100 µm. (**B**) The semi-quantification of cortical tubular injury. (**C**) The semi-quantification of outer medullary tubular necrosis. Data expressed as means ± SEM (Sham n = 3 each, 17.5 min-IRI in vehicle and SH045 n = 5 each, 20 min-IRI in vehicle and SH045 n = 4 each, respectively). Two-way ANOVA followed by Sidak's multiple comparisons post hoc test.

injury in IRI-induced AKI[12,15,16]. As a Ca^{2+}-permeable cationic channel, TRPC6 can be activated by reactive oxygen species (ROS) and mediate podocyte injury in glomerular diseases[17,18]. In addition, TRPC6 is involved in immune responses regulating transendothelial migration, chemotaxis, phagocytosis, and cytokine release[19,20]. A recent study using pharmacological blockage of TRPC6 in mice demonstrated that decreased Ca^{2+} entry due to TRPC6 contributed to reducing astrocytic apoptosis, cytotoxicity and inflammatory responses in cerebral ischemic/reperfusion insult[30]. Given that, in the present study we hypothesized that TRPC6 inhibition may also exhibit nephroprotective effects in AKI. To test this hypothesis, we applied the IRI experimental model on *Trpc6*$^{-/-}$ mice and employed a pharmacological approach of in vivo TRPC6 inhibition by SH045 and BI-749327 in wild-type (WT) mice.

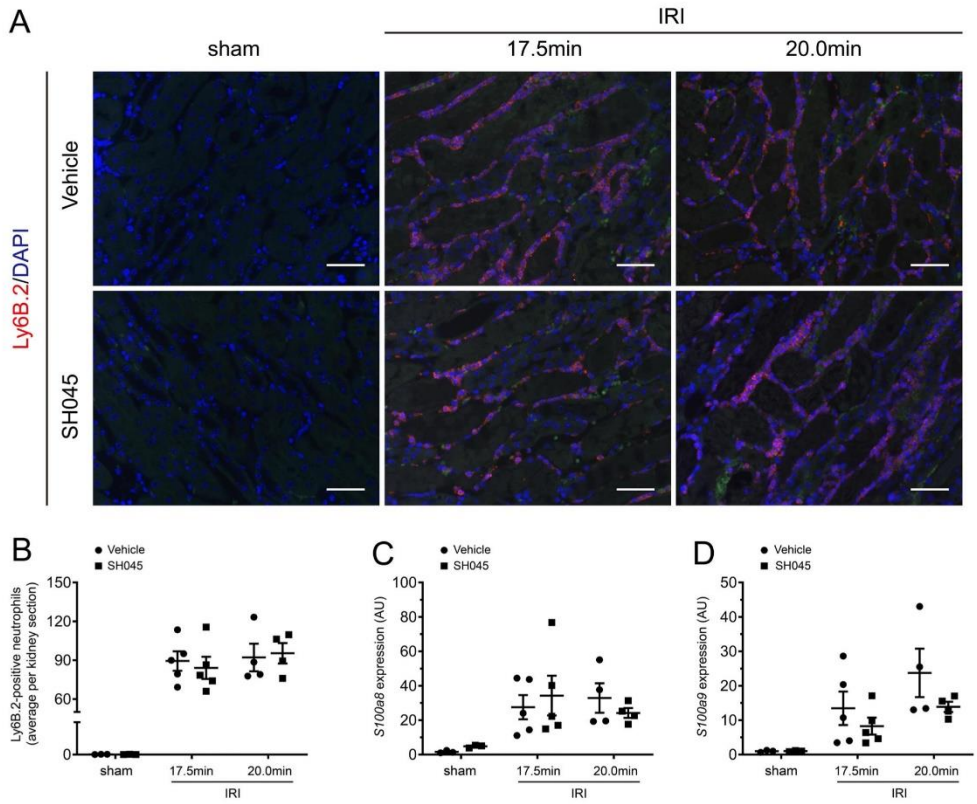

Figure 7. SH045 effects on renal neutrophils infiltration and inflammatory markers in ischemia reperfusion–induced AKI. (**A**) Representative outer medullar images of IRI-injured kidneys. Kidneys were stained with Ly6B.2 (magnification: 400×). Sham and IRI mice were treated with either vehicle or Trpc6 inhibitor (SH045). Scale bars are 100 µm. (**B**) The semi-quantification renal neutrophils infiltration. (**C**) Renal mRNA levels of S100 calcium-binding protein A8 (*S100a8*). (**D**) Renal mRNA levels of S100 calcium-binding protein A9 (*S100a9*). Data expressed as means ± SEM (Sham n = 3 each, 17.5 min-IRI in vehicle and SH045 n = 5 each, 20 min-IRI in vehicle and SH045 n = 4 each, respectively). Two-way ANOVA followed by Sidak's multiple comparisons post hoc test. AU, arbitrary units.

In our study, *Trpc6*$^{-/-}$ mice subjected to acute IRI showed no difference in renal function or tubular damage when compared to WT mice which underwent IRI. The *Trpc6*$^{-/-}$ mouse model was established as a homozygous colony, therefore we used C57BL/6 J mice as control[9]. Given this limitation of our experimental approach and to account for possible confounding genomic and non-genomic effects of other TRPC channels caused by global loss of TRPC6[31], we performed also studies using two different TRPC6 blockers SH045 and BI-749327 in C57BL/6 J WT mice to evaluate the role of TRPC6 in short-term AKI outcomes. Of note, TRPC6 and TRPC2, TRPC3, TRPC7 can form heteromeric channels with regulatory properties that are different from those of the homomeric TRPC channels[32,33]. Pharmacological inhibition allows high selectivity for inhibition of homomeric TRPC6 channels than for heteromeric channels in AKI, particularly compared to targeting TRPC6 using *Trpc6*$^{-/-}$ animals. Thus, using the pharmacological approach can precisely allow investigating the specific effects of TRPC6 homomeric channels on AKI. We used SH045 and BI-749327, and performed studies with two different ischemic times in order to have a model for mild (17.5 min) and moderate (20 min) renal damage. These two periods of reversible ischemia were based on our earlier IRI studies with mice[34–36]. However, we still found no differences with respect to serum creatinine or tubular damage between mice undergoing IRI treated with TRPC6 blocker *vs.* vehicle in both models.

Previously, we demonstrated that inhibition of tubular epithelial NF-κB activity can ameliorate IRI-induced AKI in our experimental settings[36]. Thus, our experimental conditions enable to observe beneficial effects of therapeutic interventions, i.e. kidney injury potentially can be ameliorated in our AKI mouse model. According to the reported IC_{50}-values for SH045 of 6 to 60 nM[37] and the pharmacokinetic analysis by non-compartment

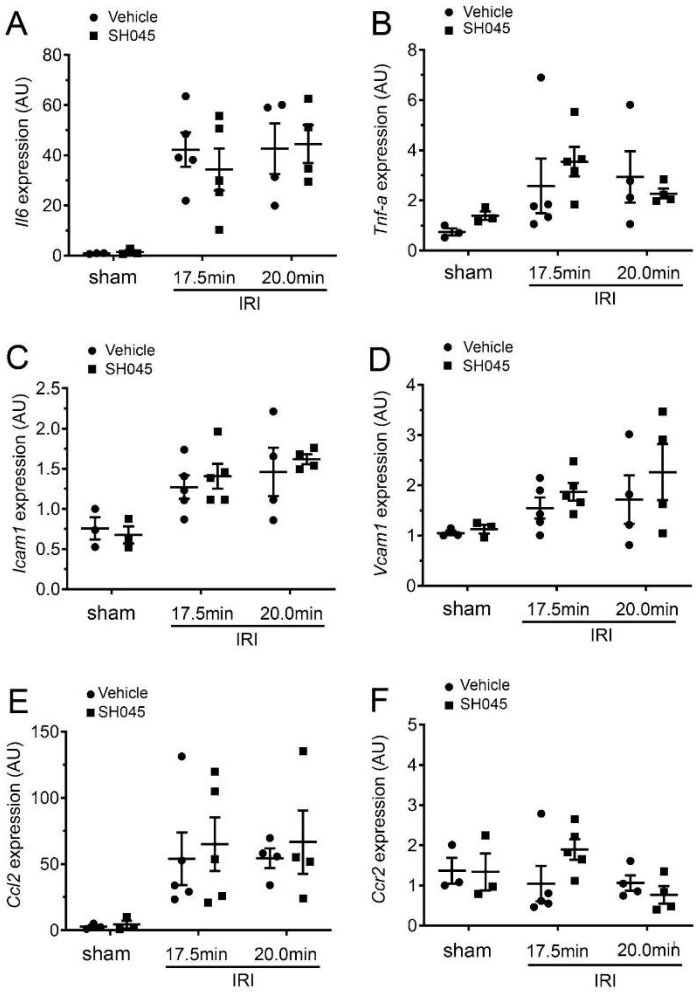

Figure 8. SH045 effects on gene expression of pro-inflammatory cytokines and chemokines after renal IRI. (**A**) Renal mRNA levels of interleukin 6 (*Il 6*), (**B**) tumor necrosis factor-α (*Tnf-α*), (**C**) intercellular adhesion molecule 1 (*Icam1*) and (**D**) vascular cell adhesion protein 1 (*Vcam1*). (**E**) Renal mRNA levels of C–C motif chemokine 2 (*Ccl2*) and (**F**) C–C motif chemokine receptor 2 (*Ccr2*). Data expressed as means ± SEM (Sham n = 3 each, 17.5 min-IRI in vehicle and SH045 n = 5 each, 20 min-IRI in vehicle and SH045 n = 4 each, respectively). Two-way ANOVA followed by Sidak's multiple comparisons post hoc test. *AU* arbitrary units.

modeling[38], SH045 can be considered as pharmacologically effective at tissue concentrations equal or higher than 22 ng/g. To confirm the drug is distributed in the kidney within the expected time, we measured the renal concentration of SH045 by using LC–MS/MS assay. As shown in Fig. 5B, the concentration of SH045 in renal tissue after 30 min post-injection (284.56 ng/g ± 144.76) was much higher than 22 ng/g. In case of BI-749327, Lin et al., reported that in concentrations of 10–30 mg/kg BI-749327 has renal protective effects in the UUO mouse model[14]. Therefore, the concentrations of pharmacological blockers used in this study were appropriate to inhibit the TRPC6 channels in vivo in our experiments.

It is now well established that inflammation plays an important role in AKI[39–42]. Shortly after endothelial or tubular epithelial cell damage, activation of resident renal inflammatory cells occurs. It leads to overproduction of cytokines, followed by recruitment and subsequent infiltration with different leukocytes subsets[43,44]. TRPC6

is expressed in a wide range of inflammatory and vascular cell types, including neutrophils, lymphocytes and endothelium[45]. During the acute phase response, TRPC6 plays a crucial role in neutrophil mobilization as it enhances chemotactic responses through increasing intracellular Ca^{2+} concentration and promoting actin-based cytoskeleton remodeling[20,46]. Indeed, after renal IRI, we found a notable accumulation of neutrophils in the region of outer medulla. However, the infiltration of neutrophils in IRI *Trpc6*$^{-/-}$ kidney was equivalent to IRI WT mice as well as in SH045-treated AKI mice compared to vehicle-treated AKI mice. Accordingly, inflammatory response including cytokines and chemokine overexpression was also similar in AKI mice with or without Trpc6 intervention. In addition, *Trpc6* was normally expressed in kidney tissue 24 h after IRI. A recent study using single-cell RNA sequencing in clusters of inflammatory cells during AKI confirms a similar renal expression of *Trpc6* mRNA in AKI versus control[47]. S100a8 and S100a9 are Ca^{2+} binding proteins belonging to the S100 family, which are constitutively expressed in neutrophils and monocytes as a Ca^{2+} sensor, participating in cytoskeleton rearrangement and arachidonic acid metabolism[48,49]. These two molecules modulate inflammatory response by stimulating recruitment of neutrophils and cytokine secretion[49]. We hypothesized that blocking intracellular influx of Ca^{2+} in neutrophils through genetical or pharmacological TRPC6 inhibition might affect the S100a8/9-mediated inflammation. However, we detected comparable renal expression of *S100a8/9* after AKI in WT and *Trpc6*$^{-/-}$ mice or in TRPC6 blockers-treated kidneys after AKI compared to respective control kidneys. Taken together, our data suggest no impact of TRPC6 to renal inflammatory reaction in the ischemic AKI.

Of note, TRPC6 is also expressed in tubular epithelial cells and plays an important role in nephron physiology[50]. Although one study showed functional importance of TRPC6 in autophagy regulation in vitro using proximal tubular cells of *Trpc6* deficient mice and TRPC6 overexpressing HK-2 cells[50], our data argue against protective effects of TRPC6 in the tubular damage of AKI in vivo. After AKI, interstitial fibrosis formation is an important part of renal repair, yet excess leads to AKI-to-CKD transition. Wu et al., and our previous study showed that inhibition of TRPC6 function either by genetic deletion ameliorates renal interstitial fibrosis[10]. Besides, another study further demonstrated an obligate function for TRPC6 in promoting fibroblast transdifferentiation[51]. Therefore, inhibition of TRPC6 may have effects on renal fibrogenesis during AKI-to-CKD transition, and the beneficial effects of TRPC6 inhibition seen in the UUO model most likely involve fibroblast activation and transdifferentiation. Since its differentiation is an expected long-term outcome of AKI[52], this might explain the lack of *Trpc6*$^{-/-}$ effects on the short-term outcome of AKI in present study. In addition, renal microvascular dysfunction is considered playing critical role in acute kidney injury[53]. Due to unchanged myogenic tone after TRPC6 modulation in vascular smooth muscle cells using isolated perfused kidneys, it is unlikely that a vascular component represents a protective mechanism of renal TRPC6 inhibition.

In summary, the present study shows that TRPC6 inhibition has no effects on short-term outcome of AKI. Our results improve the understanding of the role of TRPC6 role in kidney diseases. Future studies should investigate if TRPC6 could have a protective role on long-term outcome of AKI. Our results may have clinical implications for safety and long-term outcome of humans with *TRPC6* gene variations leading to familial forms of FSGS, with respect to the response of the kidney to acute ischemic stimuli.

Materials and methods

Animals. Male *Trpc6*$^{-/-}$ mice (n = 10, homozygosity in a mix 129 Sv:C57BL/6J background) and wild-type (WT, C57BL/6J) control mice (n = 9) were used. *Trpc6*$^{-/-}$ mice have been generated and characterized previously[9,31]. For the pharmacological study WT mice (C57BL/6J, Jackson Laboratory) were used. Mice were held in specific-pathogen-free (SPF) condition, in a 12:12-h light–dark cycle, and with free access to purified food (E15430-047, Ssniff, Soest, Germany) and water. Experiments were approved by the Berlin Animal Review Board, Berlin, Germany (No. G0178/18) and followed the restrictions in the Berlin State Office for Health and Social Affairs (LaGeSo)[54]. All experiments were performed in accordance with ARRIVE guidelines[55].

Renal IRI model. Renal IRI was induced as described earlier[34]. Briefly, male mice (aged 14–18 weeks) were anaesthetized by isoflurane (2.3%) in air (350 ml/min). Preemptive analgesia with buprenorphine (0.2 mg/100 g) was used[56]. Mice were operated individually to ensure similar exposure to isoflurane[57]. Body temperature was maintained at 37 °C and monitored during surgery. Ischemia was induced after right–sided nephrectomy by clipping the pedicle of the left kidney for 17.5 or 20 min with a non–traumatic aneurysm clip (FE690K, Aesculap, Germany). Reperfusion was confirmed visually and the abdomen and the skin were sutured separately with a 5/0 braided-silk suture. After surgery mice had free access to water and chow. Body–warm sterile physiological saline solution (1 ml) was applied subcutaneously to every mouse. Sham operation was performed in a similar manner, except for clamping the renal pedicle. Twenty–four hours after reperfusion, mice were sacrificed by overdose of isoflurane and additionally cervical dislocation, and kidney and blood samples were collected for further analysis. The kidneys were divided into three portions. Upper pole of the kidney tissue was frozen and used for measurement of SH045 concentration. Middle part of kidney was immersed in 4% phosphate–buffered saline (PBS)–buffered formalin for histology, and the other left tissue was snap–frozen in liquid nitrogen for RNA preparation.

TRPC6 blocker. Larixyl-6-N-methylcarbamate, also called SH045, is a compound with high affinity and subtype selectivity toward TRPC6 described by Häfner et al.[22]. SH045 was initially dissolved in DMSO (ratio of DMSO to vehicle is 0.5%) and then in 5% Cremophor EL® solution with 0.9% NaCl and used for intravenous injection (2 mg/kg) 30 min before IRI surgery in the pharmacological studies with WT mice. BI-749327 is an orally bioavailable TRPC6 antagonist as reported[14]. BI-749327 (MedChemExpress, New Jersey, USA) was dissolved in DMSO and then suspended in corn oil (final ratio of DMSO to corn oil is 5%). For administration, 30 mg/kg of the final dose was delivered to mice via oral gavage 60 min before IRI surgery.

Blood measurements and drugs. To allow repeated blood measurements of sodium, potassium, chloride, ionized calcium, total carbon dioxide, glucose, urea nitrogen, creatinine, hematocrit, hemoglobin and anion gap within a short time interval in the same mouse, 95 µL blood was taken from the facial vein and parameters were measured using an i-STAT system with Chem8 + cartridges (Abbott, USA).

Quantitative real-time (qRT)-PCR. qRT-PCR was performed as described previously[9]. Briefly, Total RNA from snap-frozen kidneys were isolated using RNeasy RNA isolation kit (Qiagen, Australia) according to manufacturer's instruction after homogenization with a Precellys 24 homogenizator (Peqlab, Germany). RNA concentration and quality were determined by NanoDrop-1000 spectrophotometer (Thermo Fisher Scientific, USA). Two micrograms of RNA were transcribed to cDNA (Applied Biosystems, USA). Quantitative analysis of target mRNA expression was performed with qRT-PCR using the relative standard curve method. TaqMan and SYBR green analysis was conducted using an Applied Biosystems 7500 Sequence Detector (Applied Biosystems, USA). The expression levels were normalized to 18S. Primer sequences are provided in Supplementary Table S1.

Histology and analysis. Formalin-fixed, paraffin-embedded sections (2 µm) of kidneys were subjected to Periodic acid Schiff (PAS) stain according to the manufacturer's protocols (Sigma, Germany). Semi-quantitative scoring of tubular damage was performed in a blinded manner in those 12 to 15 images at 200 × magnification per sample. Acute tubular injury (ATI) was observed in this study to assess the reversible tubular damage due to ischemia. The histologic features of ATI included one or more of the following lesions: tubular epithelial swelling with lucency of the cytoplasm, loss of brush border, luminal dilatation with simplification of the epithelium, and cytoplasmic vacuolization. Acute tubular necrosis (ATN) was also evaluated, which was indicated by patchy loss of tubular epithelial cells with resultant gaps and exposure of denuded basement membrane, evidence of cellular regeneration, as well as obliterated tubular hyaline and/or granular casts. The histological findings were graded from 0 to 3 according to the distribution of lesions: 0 = none; 1 = < 25%; 2 = 25–50%; 3 = > 50%[56]. The total score was calculated as sum of all morphological parameters. Tubular damage and tubular necrosis were quantified by an experienced renal pathologist who was unaware of Trpc6 genotypes or of treatment.

HPLC and tandem mass spectrometric method (LC–MS/MS). Concentration of SH045 in kidney homogenates was measured as previously described[23]. In summary, analyses were performed with an Agilent 1260 Infinity quaternary HPLC system (Agilent Technologies, Germany) consisting of a G4225A degasser, G1312B binary pump, G1367E autosampler, G1330B thermostat, G1316A column oven and G4212B diode array detector, coupled to a tandem QTRAP 5500 hybrid linear ion-trap triple quadrupole mass spectrometer (AB SCIEX, Canada). Data were acquired and processed using Analyst software (Version 1.7.1, AB SCIEX). Linear regressions and calculations were done using Multiquant software (Version 2.1.1, AB SCIEX). Lower limit of quantification (LLOQ) and accuracy were determined for quantification of SH045 effects in kidney and both revealed as high as reported for plasma by Chai et al.[38].

Immunofluorescence and analysis. We performed immunofluorescence similarly as previously described[34]. Two-µm thick sections of IRI-injured kidneys were post-fixed in ice-cold acetone, air-dried, rehydrated and blocked with 10% normal donkey serum (Jackson ImmunoResearch, America) for 30 min. Then sections were incubated in a humid chamber overnight at 4 °C with rat anti-Ly6B.2 (Gr1) (1:300; MCA771G; Bio-Rad AbD Serotec, Germany). The bound anti-Ly6B.2 antibody was visualized using Cy3-conjugated secondary antibody (1:500; Jackson ImmunoResearch, America) by incubating the sections for 1 h in a humid chamber at room temperature. Positive cells were counted in the outer medulla on ten non-overlapping view fields at 400 × magnification and mean cell numbers were taken for analysis.

Isolated perfused kidneys. Isolated kidneys were perfused in an organ chamber using a peristaltic pump (Instech, USA) at constant flow (0.3–1.9 ml/min) of oxygenated (95% O_2 and 5% CO_2) physiological salt solution (PSS) containing (in mmol/L) 119 NaCl, 4.7 KCl, 1.2 KH_2PO_4, 25 $NaHCO_3$, 1.2 Mg_2SO_4, 11.1 glucose, 1.6 $CaCl_2$). Hyperforin (Sigma-Aldrich, USA), BI-749327, and SH045 were dissolved in DMSO. Before application, the aliquots were dissolved 1:1000 in PSS. So, the final concentration of DMSO did not exceed 0.1%. The concentrations of hyperforin, BI-749327, SH045 were 10 µM, 100 nM, and 100 nM, respectively. We used PSS as a solvent for Ang II (LKT Laboratories Inc., USA) and applied it at 10 nM concentration. Drugs were added to the perfusate. Perfusion pressure was detected using pressure transducer after an equilibration period of 60 min. Powerlab system (AD Instruments, Colorado Springs) was used for data acquisition and analysis. Ang II-induced pressor effects were normalized to the maximal pressor effect obtained with KCl (60 mmol/L)[58]. The composition of 60 mM KCl (in mmol/L) was 63.7 NaCl, 60 KCl, 1.2 KH_2PO_4, 25 $NaHCO_3$, 1.2 Mg_2SO_4, 11.1 glucose, and 1.6 $CaCl_2$.

Statistics. Statistical analysis was performed using GraphPad 5.04 software. Study groups were analyzed by one-way ANOVA using Turkey's post-hoc test or two-way ANOVA using Sidak's multiple comparisons post hoc test. Data are presented as mean ± SEM. P values < 0.05 were considered as statistically significant.

Experimental statement. All methods were carried out in accordance with relevant guidelines and regulations.

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Author contributions
All authors planned and designed experimental studies. Z.Z., D.T., and T.U.P.B. performed qPCRs, histological evaluation, analyzed data and drafted the manuscript. C.E. and J.S. performed the isolated kidney perfusions. U.K. and M.S. supplied the TRPC6 Blocker and provided pharmacokinetic measurements for its use in vivo. X.C. and F-A.L. measured the concentration of SH045 in vivo. K.W. supervised the histological analysis. G.N. performed murine surgery and collected blood and tissues. M-B.K. performed renal PAS and immunofluorescence staining. B.N., M.G. and L.M. supervised the experimental work. All authors revised the manuscript critically for important intellectual content. All authors agree to be accountable for all aspects of the work in ensuring that questions related to the accuracy or integrity of any part of the work are appropriately investigated and resolved. All authors made substantial contributions to conception, design, drafting and completion of the article.

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4. Interferon-γ signaling inhibition ameliorates angiotensin II-induced cardiac damage

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Hypertension is the major cardiovascular disease risk factor, with an estimated half of the cardiovascular events attributed to it.[109] According to a current estimate (including hypertension-associated chronic kidney disease (CKD)), this reflects 7.7–10.4 million annual deaths worldwide.[110] Hypertension leads to target organ damage, ultimately leading to organ failure and death.[111] In the last decade, ample evidence suggests that angiotensin II (Ang II), one of the effector molecules of the renin-angiotensin-aldosterone system (RAAS), plays a central role in mediating target-organ damage. The first practicable model was introduced by Goldblatt.[112] He described that renal ischemia is sufficient to induce persistently elevated systolic blood pressure; later, Ang II was identified as the mediator.[113] Accordingly, drugs interfering with the RAAS system effectively reduce blood pressure and prevent target organ damage in patients with hypertension.[114] More importantly, they reduce cardiovascular mortality in patients with various types of cardiovascular disease.[115-118]

To investigate the mechanism of Ang II-induced organ damage, animal models with high Ang II blood levels were generated.[46,119] Soon, it was discovered that inflammatory mechanisms are principal mediators of Ang II-induced target organ damage, and anti-inflammatory agents or immunosuppressants can reduce it.[120-122] In a milestone paper Guzik et al. used mice lacking T and B cells and showed that these mice develop only blunted hypertension upon Ang II infusion. In these mice, hypertensive response to Ang II could be achieved by restoring the T cell population, whereas by restoring B cells not. These findings suggested that T cells are not only a mediator of end-organ damage but essential for developing hypertension itself. This work, however, did not address which T cells are responsible for the observed effect.

As a previous finding suggested a disturbed balance between different T helper cell (Th) subsets,[123] we decided to investigate the role of Th1 and just recently described interleukin (IL)17A producing Th17[124] subsets in angiotensin-induced renal and cardiac damage. Th1 cells are characterized by the secretion of interferon-gamma (IFNγ), and the generation of

pathological Th17 cells is IL23-dependent.[125-127] Therefore, we used IFNγ receptor (IFNγR) knockout (KO) mice[128] or treated outbred NMRI mice with neutralizing antibodies against the IL23 receptor (IL23R) antibody or against IL17A. As control mice, 129S6/SvEvTac mice (genetic background of the IFNγR-KO mice) were used as the strain existed only in a homozygous form for the null mutation of the IFNγR gene, or NMRI mice were treated with the respective isotype IgG1 control antibodies. Subcutaneous osmotic micropumps delivered Ang II for 2 weeks. Antibodies were given intraperitoneally twice during the experiment: on the day of micropump implantation and one week after. All experimental mouse groups developed similarly high blood pressure upon Ang II. However, IFNγR-KO mice presented somewhat lower mean arterial blood pressure than wild-type mice, but it did not reach statistical significance in our case. Two years later, Kamat et al. could show a blunted rise in Ang II-induced high blood pressure in IFNγ-KO mice.[129] Nevertheless, Ang II-treated IFNγR-KO mice exhibited lower expression of adhesion molecules on the vascular endothelium, reduced T cell and macrophage infiltration, and less perivascular and interstitial fibrosis in the heart, in comparison to Ang-II-treated wild-type mice. On a functional level, this histological picture was associated with a drastically decreased rate of inducible, reproducible non-sustained ventricular arrhythmias as assessed by *in vivo* electrophysiological studies. Supporting the result of the electrophysiological studies, the cardiac expression of connexin 43 (one of the main gap junction proteins in the cardiac ventricle connecting adjacent cells) was restricted to the microscopic structures of the heart, the so-called lines of Eberth or intercalated disc regions in Ang II-treated IFNγR-KO mice. Whereas in Ang II-treated wild-type mice, connexin 43 was dislocated. Treatment neither by IL23R nor by IL17A significantly influenced Ang II-induced hypertension or cardiac damage.

We next analyzed kidney function and renal histology to assess hypertensive renal damage in the experimental groups. Hypertensive IFNγR-KO mice exhibited - similarly to the heart - a lower number of infiltrating T cells and macrophages and less fibrosis compared to Ang II-treated wild-type mice. This was associated with lower induction of the renal damage marker neutrophil gelatinase-associated lipocalin and, on the functional level, with a lower reduction of glomerular filtration rate in Ang II-treated IFNγR-KO mice. In contrast to this ameliorated hypertensive renal damage phenotype, hypertensive IFNγR-KO mice had almost five times higher albuminuria than their hypertensive wild-type controls. Staining of renal tissue of Ang II-treated IFNγR-KO mice against the podocyte-specific protein, Wilms' tumor 1,[130] revealed fewer podocytes than the respective wild-type control group, indicating accelerated podocyte death. Moreover, staining against nephrin, a structural component of the slit diaphragm,[131] and against synaptopodin, an actin microfilament-associated protein in the podocyte foot

processes[132] showed much weaker staining in hypertensive IFNγR-KO mice when compared to renal staining of hypertensive wild-type mice. Earlier studies suggested that IFNγ might interfere with autophagy.[133,134] Therefore, we stained renal tissue against the autophagosomal marker LC3B.[135] The staining revealed significant LC3B accumulation in Ang II-infused IFN-γR KO mice podocytes. In contrast, Ang II-infused wild-type mice had only a low percentage of LC3B positive podocytes. Blockade of IL23 signaling or neutralizing IL17A did not affect hypertensive renal damage development.

We concluded that inhibiting IFN-γ signaling, but not IL23 or IL17 signaling, protects against Ang II-induced cardiac damage and consequent arrhythmias. In hypertensive renal damage, IFN-γ signaling has opposing effects on renal function, depending on the cell type where it is expressed. The absence of IFN-γR signaling preserves renal function, whereas it leads to albuminuria as podocytes seem to require intact IFN-γ signaling to maintain integrity and functionality.

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5. B-cell lymphoma/leukemia 10 (Bcl10) and angiotensin II-induced kidney injury

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As described in the previous section, angiotensin (Ang) II is crucial in developing hypertensive kidney injury[136-138], and immune cells are a central feature of this process.[51,120-122,139] The transcription factor nuclear factor kappa-light-chain-enhancer of activated B cells (NF-κB) is a main regulator of immune cell function.[140,141] Ang II can stimulate the intracellular formation of reactive oxygen species by activating subunits of the membrane-bound nicotinamide adenine dinucleotide phosphate oxidase and also by increasing its formation in the mitochondria and thereby inducing NF-κB.[142] Additionally, immune cells express the angiotensin II type 1 receptor (AT1R) and can modulate the immune cells' function under certain circumstances.[143,144] Yet, the signaling pathway from the AT1R to NF-κB was incompletely understood.

T and B cell receptors are complex structures that operate with accessory proteins and activate multiple signaling pathways to elicit a proper immune response. One of the central signaling pathways T and B cell receptors initiate involves the activation of specific protein kinase C isoforms, which is necessary to activate the NF-κB.[145] How protein kinases however signal to NF-κB was unknown.[145] It was not until the beginning of the new century when the mechanism was cleared. The signalosome consists of three major signaling molecules: (i) caspase recruitment domain 11 (also known as CARMA1), which integrates the upstream signal of activated protein kinase C with downstream factors; [146-149] (ii) B-cell lymphoma/leukemia 10 (Bcl10) an intermediate bridging factor;[150,151] and the effector protein (iii) mucosa-associated lymphoid tissue lymphoma translocation protein 1 (MALT1), which ultimately stimulates the IκB kinase complex (IKK) complex.[152,153] Since CARMA1 is lymphocyte-specific, the signalosome - and therefore the NF-κB activating pathway - is exclusive to cells of the immune system.[147,149] However, the expression profile of CARMA3, another member of the CARMA family, is much broader. In 2007 McAllister-Lucas and colleagues described a novel pathway - including the CARMA3-Bcl10-MALT1 (CBM) signalosome – as a mediator of Ang II-dependent activation of NF-κB.[154]

We showed earlier that lacking Bcl10, the intermediate bridging factor of the signalosome, ameliorates Ang II-induced cardiac inflammation, cardiac fibrosis, and electrical remodeling as a result of lower NF-κB activation by Ang II.[155] With the help of bone marrow transplantation studies, we further showed that Bcl10, of both immune cells and of cardiac origin, contributes to the ameliorated phenotype.[155] Based on data and partly tissue samples from this study, we tested the hypothesis that lacking Bcl10 reduces Ang II-induced hypertensive renal damage. For this purpose, we used mice lacking Bcl10[156] and performed additional experiments. Uninephrectomized Bcl10-deficient (Bcl10KO) and C57BL/6 wild-type mice (genetic background of the Bcl10KO mice, since Bcl10KO mice existed only in a homozygous form for the null mutation), as well as mice that underwent allogeneic kidney transplantation, were infused with Ang II via subcutaneous osmotic micropumps for 2 weeks and received 1% NaCl (ad libitum) in the drinking water. Removing one kidney before delivery of Ang II (after surgery, mice had at least 1 week recovery time) and salt-containing drinking water were necessary because C57LB/6 mice are more resilient to renal injury than other mouse strains.[157,158]

On the one hand, we detected less fibrosis and T cell and macrophage infiltration in the kidneys of Ang II-treated Bcl10KO mice compared to Ang II-treated wild-type mice. On the other hand, we detected a higher level of albuminuria, which was associated with a higher renal expression of tubular damage marker neutrophil gelatinase-associated lipocalin and kidney injury molecule 1. Elevated albuminuria was associated with aggravated podocyte injury, as evidenced by the stain of nephrin, a slit diaphragm protein, and a lower number of Wilms' tumor 1 (podocyte-specific marker) positive cells in the glomerulus. Ultimately, Ang II-treated mice lacking Bcl10 showed deteriorated renal function as indicated by higher serum creatinine levels compared to Ang II-treated wild-type mice. To further investigate this seemingly contradictious phenotype of hypertensive renal injury and to separate the actions of Bcl10 in the kidney from the global in all tissues, we performed allogenic kidney transplantation studies, whereby wild-type or Bcl10KO kidneys were transplanted into wild-type recipients. After recovery from the surgery, the mice underwent the same experimental procedure as mice from the first studies (uninephrectomy, saline solution, and Ang II treatment, though this case only for a week). Those wild-type mice transplanted with Bcl10KO kidneys showed higher renal expression of neutrophil gelatinase-associated lipocalin and fewer podocytes. However, the level of immune cell infiltration and renal fibrosis was comparable in both wild-type and Bcl10 recipient wild-type mice. This suggests - together with the phenotype of global Bcl10KO animal studies - that the ameliorated fibrosis can be attributed to a deficient, Bcl10-dependent NF-κB

signaling of immune cells and the greater podocyte damage and the consequent tubular damage to the lack of Bcl10 in renal cells.

Finally, we also analyzed the cardiac hypertrophy in the allogenic transplantation groups. Surprisingly, upon Ang II treatment, wild-type mice with Bcl10KO kidneys transplanted developed markedly reduced cardiac hypertrophy compared to wild-type kidney transplanted wild-type mice. In a similar experimental setting, but with AT1RKO mice, a similar cardiac phenotype was observed: mice lacking the AT1R only in the kidney developed significantly less cardiac hypertrophy than wild-type mice transplanted with wild-type kidneys.[159] In that manuscript, Crowley et al. concluded that renal AT1 receptors are indispensable for the development of And II-dependent cardiac hypertrophy and hypertension. This similarity with our renal Bcl10KO phenotype supports the role of Bcl10 in Ang II signaling. Nevertheless, further studies are needed to confirm this notion.

In summary, we provide evidence that Bcl10, the bridging molecule of the recently discovered CBM signalosome, is a crucial component of mediating Ang II-induced induced renal immune cell infiltration and fibrosis. Furthermore, we uncovered a novel role for Bcl10 in maintaining podocyte integrity. Supporting ameliorated Ang II-induced cardiac hypertrophy in earlier findings with mice deficient for the AT1R in the kidney only, we found that lacking Bcl10 solely in the kidney resembles this phenotype. This supports the role of the CBM signalosome in renal AT1R signaling, a pathway that was described in the liver only.[154] However, to support the latter statement, further studies are needed to prove this hypothesis undisputedly.

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III. Discussion

Nephrology is a relatively young medical subspecialty that is continuously evolving.[4] As consensus definitions of acute and chronic renal diseases were established, the global challenge these diseases pose became clear. AKI is a common complication in hospitalized patients affecting approximately 10–15% of them and almost 50% of those patients in the intensive care unit.[7,8] Whereas CKD, according to the 2010 Global Burden of Disease study, ranked 18th in the list of causes of the total number of deaths and affected an estimated 800 million people worldwide.[160,161] Since both entities have limited treatment options (mainly supportive or reduced to treating complications and consequences of renal function loss), there is an urgent need to find therapeutic targets.

In the presented experimental works, new mouse models were developed, and existing ones were applied to discover and investigate novel signaling pathways and mediators in the kidney which could influence the outcome of ischemic AKI and inflammatory signaling pathways as novel mediators of hypertension-induced chronic renal damage were investigated.

Members of the NF-κB transcription factor family form hetero- or homodimers that can regulate gene transcription through two distinct activation pathways: canonical and non-canonical. The canonical (also known as classical) pathway dynamically governs the response to various external stimuli, including ligands relevant in the context of AKI, such as multiple cytokine receptors or the TNF receptor superfamily members. Previous experimental studies applied global NF-κB inhibition and could show that this strategy can alleviate renal damage; however, studies with cell- or tissue-specific NF-κB inhibition have demonstrated that the effects of NF-κB vary significantly in different cell types.[162] In our work, we have addressed the role of NF-κB in tubular epithelial cells in ischemic AKI. We used the Cre-loxP system and expressed the Cre enzyme under the Emx1 promoter, which we have shown to be expressed in the renal tubular epithelium. These mice were mated with mice bearing the floxed NF-kB superrepressor, IκBαΔN, a genetically modified version of NF-kB inhibitor and main canonical pathway target gene IκB to obtain mice with reduced NF-κB activity (Emx1-ΔN) in the renal tubular cells. In agreement with previous studies[163,164] where NF-κB was inhibited globally, we also observed reduced immune cell infiltration in the injured kidneys of mice with tubular specific NF-κB inhibition compared to injured kidneys of wild-type littermates, highlighting the role of epithelial cells in leukocytes recruitment and inflammatory response after ischemic AKI. We also found fewer apoptotic cells in the injured kidneys of the Emx1-ΔN mice in comparison to the injured kidneys of wild-type mice, which finding we verified in *in vitro* experiments in a

hypoxia-mimetic model using $CoCl_2$ and proximal tubular cell cultures from wild-type mice and mice overexpressing IκBαΔN. Although NF-kB is mainly involved in apoptosis suppression[165], these data argue for an apoptotic role of NF-kB in the context of ischemic renal damage. An additional mouse model was used, which expressed the luciferase gene under the control of NF-κB, showing that renal ischemia leads to significant NF-κB activation already 12 hours and peaking 3 days after insult. Microarray analysis of injured kidneys of Emx1-ΔN mice 24 hours after ischemia revealed 240 genes differentially downregulated compared to damaged kidneys of wild-type mice. Of these, known NF-kB target genes were overrepresented, as demonstrated by gene-set enrichment analysis, such as cytokines or chemokines, cell adhesion molecules, and stress response genes, including the chemokine (C-X-C motif) ligand 1 and 2 and vascular cell adhesion molecule 1, all which are involved in post-ischemic recruitment of inflammatory cells.[166-168] Of clinical importance, preoperative administration of (low dose) aspirin, a well-known anti-inflammatory drug and NF-κB inhibitor,[169] was found recently to be associated with reduced hemodialysis requirements and reduced postoperative length of hospital stay without significant increase of bleeding events.[170] Thus, our data argue for a clinically targetable role of renal NF-kB activation in ischemic renal injury.

Next to nitric oxide and carbon monoxide, H_2S emerged 30 years ago as a third gasotransmitter. While many experimental works have provided evidence that H_2S is protective in diverse kidney damage models,[81,171,172] no experimental work focused on the (pathos)physiological role of H_2S. In the kidney, H_2S is produced mainly by two enzymes, cystathionine β-synthase and cystathionine γ-lyase (CTH). Here we investigated the role of CTH-derived H_2S in AKI. We used $Cth^{-/-}$ mice on a backcrossed C57BL/6J background,[86], whereas another $Cth^{-/-}$ mouse[87] model exists on a mixed genetic background. Of note, although the same gene was targeted in both mice, different exons were deleted, and phenotypical differences of unclear reasons exist. Additionally, as our experiments were still running, Bos et al. published their results using the other mouse model in an AKI model.[92] Similarly to their findings, we also found reduced renal expression (on gene and protein level) of both CTH and CBS after renal IRI. Additionally, no difference in post-ischemic infiltration of neutrophil granulocytes or macrophages could be observed in both experimental works. On the other hand, whereas we did not detect any differences in renal IRI damage between $Cth^{-/-}$ and wild-type or even in heterozygous $Cth^{+/-}$ littermates, Bos et al. found aggravated kidney damage in their $Cth^{-/-}$ mice after IRI, which was associated with increased mortality. The reasons for this discrepancy are possibly multifaceted. It includes baseline differences of the two mouse models (e.g., their $Cth^{-/-}$ mice develop an age-dependent elevation in blood pressure[87] whereby blood pressure starts to elevate by 10 mmHg already at the age of 7 weeks

and sex-related hyperhomocysteinemia, whereas the $Cth^{-/-}$ mice we used do not). Moreover, the surgical procedure was different, although serum creatinine levels in both studies were similar 24 hours after IRI. Finally, CTH deletion led to a 50% reduction in renal H_2S concentration in our mouse model, whereas they observed a decrease of over 90%. Our findings that $Cth^{-/-}$ mice do not exacerbate IRI-induced renal damage is in contrast to the previous study where CTH was inhibited chemically by dl-propargylglycine (50 mg/kg intraperitoneally, one hour before surgery) and led to reduced recovery from renal IRI in a rat bilateral ischemia model.[31] However, the same compound (5 mg/kg intraperitoneally, twice a day for four consecutive days) had nephroprotective effects in the cisplatin model of AKI in rats and in the adriamycin-induced nephrotoxicity in rats (50 mg/kg intraperitoneally, injected 2 hours after drug application).[173,174] Indeed, proinflammatory roles of H_2S have also been shown, suggesting that it might accelerate inflammatory responses.[175] Thus, future studies are needed to elucidate the role of endogenous H_2S in renal IRI.

Renal damage caused by acute ischemic injury often progresses into chronic tissue damage, ultimately leading to CKD. Although channelopathies are rare causes of CKD, identifying ion channels in disease can open new avenues to treat renal conditions. After all, approximately 15% of drugs used presently target ion channels.[176] We aimed to investigate the TRPC6 channel, a non-selective cation channel, which has been recently identified as a cause of a familiar form of FSGS as a potential druggable target to alleviate the immediate outcome of AKI.[34] In the context of kidney diseases, the role of TRPC6 has been investigated in renal fibrosis caused by urinary obstruction, in murine diabetic nephropathy models, and in autoimmune nephritis models, as reviewed recently.[177] Here, we investigated if lacking or inhibiting TRPC6 could be favorable in reducing renal damage in AKI. First, we used the $TRPC6^{-/-}$ mouse model that was generated and characterized earlier.[96] Since this mouse lineage was kept homozygous in a mixed 129Sv:C57BL/6J genetic background, we decided to use C57BL/6J mice as control wild-type mice. Upon IRI, both mice developed a similar degree of renal function decreased as mirrored by similarly elevated serum creatinine, blood urea nitrogen, and potassium levels. Renal pathology also showed comparable renal damage in wild-type and TRPC6-/-, which underwent IRI and a similar degree of neutrophil granulocyte infiltration. This finding was somewhat unexpected as we used global knockout animals, and previously others have shown that TRPC6 channels play a role in immune cell migration and chemotaxis.[105,106] Additionally, as we were finishing these experiments, Hou et al. using another TRPC6 knockout mouse linage (129SvEv), showed significantly ameliorated renal damage after IRI.[108] Nevertheless, their TRPC6 knockout mice were probably also kept homozygous since they used not littermates but 129SvEv mice as control wild-type mice. Additionally, targeting TRPC6

channels has been shown earlier to alleviate tissue damage in other ischemic models, such as focal cerebral ischemia.[107] Because of this, we decided to target TRPC6 pharmacologically by applying two different specific antagonists, SH045 and BI-749327. We even introduced a group of mice with shorter renal ischemia time (although the previously applied 20-minute ischemia time also did not lead to irreversible renal damage). We measured and confirmed biologically sufficient concentrations of SH045 in renal tissues and used earlier verified concentrations of BI-749327.[39] However, similarly to the experimental data with TRPC6$^{-/-}$ mice, we could not observe any effect of TRPC6 inhibition on the extent of renal damage, blood parameters or cellular infiltration in comparison to vehicle-treated mice. Of note, we also used C57BL/6J mice for the pharmacological studies. Therefore the genetic background might explain discrepancies between our findings with TRPC6$^{-/-}$ mice and those published by others.[108] Nevertheless, in renal fibrosis models both our TRPC6$^{-/-}$ mice and SH045 treatment in the same dosage significantly reduced the extent of fibrosis and cellular infiltration and decreased the mRNA expression of pro-fibrotic markers.[40,178] Thus further studies should test the hypothesis if TRPC6 inhibition might be protective on the long-term consequences of ischemic AKI.

Chronic renal disease progresses into ESRD. Next to diabetes, hypertension is the leading cause of ESRD.[42] Inflammatory cells have long been described as an essential mediator of hypertensive organ damage. However, only a recent study identified T cells as crucial players in the development of hypertension and considered them as mediators only.[51] In the fourth work, we investigated the role of T cell subtype Th1 and Th17 in the development of hypertensive renal damage.[179] We used this time next to a genetic mouse model (IFNγR KO mice to investigate Th1 cells),[128] and a neutralizing antibody strategy against the IL23 receptor (IL23R) and IL17A, to inhibit the generation and function of the Th17 cells, respectively. Hypertension was induced by continuous Ang II infusion by osmotic minipumps. We found that lacking the IFNγR signaling pathway, but not IL23R or IL17A antibody treatment, improved Ang II-induced cardiac and renal end-organ damage without affecting the development of Ang II-induced high blood pressure. However, a complete IL17 inhibition by knocking out the IL17A gene led to reduced blood pressure response upon Ang II infusion in mice lacking the IL-17A gene.[180] Nevertheless, blunted Ang II responsiveness could only be observed starting at 3 weeks after Ang II treatment.[180] In contrast to Th17 inhibition, Ang II-infused IFNγR KO (Th1) mice showed ameliorated cardiac hypertrophy and electric remodeling rendering them less prone to develop non-sustained ventricular arrhythmias than Ang II-infused control mice. In line with the ameliorated cardiac damage phenotype, renal damage was also less apparent, and the glomerular filtration rate was more preserved. Nonetheless, Ang II led to higher albuminuria and reduced podocyte number, as well as synaptopodin and

nephrin expression, which we explain partly by the impaired podocyte autophagy we detected in these mice. In line with our observation, IFNγ KO mice or control mice injected with IFNγ-neutralizing antibody developed worse albuminuria and glomerular pathology than control mice.[181] These data shed light on the contribution of Th1 and Th17 immune cells in the development of Ang II-induced target-organ damage.

Ang II mediates end-organ damage partly by activating NF-κB through binding to its receptor AT1.[54] Here, AT1R is expressed not only on "classical" target cells such as endothelial cells, vascular smooth muscle cells, or specific tubular cells of the kidney but on immune cells too.[55,182] However, the exact molecular pathway of how AT1R signals to NF-κB in different cell types was unclear until 2007, when a signalosome containing the bridging factor Bcl10 was described in both immune and nonimmune cells.[154] We investigated this pathway in an Ang II-induced hypertension model using Bcl10 KO mice in the fifth work.[183] Here, we found that global Bcl10 deficiency protects against Ang II-induced cellular infiltration and renal fibrosis; however, it aggravates renal damage manifesting in exacerbated albuminuria and tubular damage. By performing allogeneic kidney transplantation, we could conclude that Bcl10 in the kidney is necessary to limit Ang II-induced renal cellular damage, and Bcl10 in immune cells mediates cell infiltration and renal fibrosis. As a matter of fact, Bcl10 KO mice exhibit an impaired immune response to various stimuli.[156] In this regard, our findings with global Bcl10 KO mice are in agreement with various other immunodeficient mouse models where an ameliorated end-organ damage was reported upon Ang II-induced hypertension.[120,184] Indeed, Ang II mediates the recruitment of immune cells into the kidney by expression of NF-κB regulated adhesion molecules, cytokines, and chemokines, ultimately leading to renal fibrosis.[47,55,185] A more surprising finding was the aggravated podocyte damage and consequently higher albuminuria upon Ang II treatment in Bcl10 deficient mice compared to control mice. As podocyte-specific inhibition of NF-κB was protective in a mouse model of adriamycin-induced nephropathy,[186] we expected a similar effect in our model. Podocytes have a unique cytoarchitecture with a precise and dynamic organization of actin filaments.[187] Bcl10 also plays a role in F-actin cytoskeleton remodeling, independent of NF-κB signaling in immune cells.[188,189] Indeed, we have shown earlier that both *in vitro* and *in vivo* macrophage chemotaxis, a process which requires actin rearrangement, toward monocyte chemotactic protein-1 is severely impaired when lacking Bcl10.[179] We, therefore, speculate that Bcl10 has a similar role in podocytes, which would explain a faulty remodeling upon Ang II. Another interesting finding was that control mice transplanted with Bcl10 KO kidney developed significantly ameliorated cardiac hypertrophy in comparison to control mice transplanted with control kidney. This phenomenon resembled the transplant studies using AT1R KO mice

whereby control mice transplanted with AT1A receptor deficient kidneys were also protected against cardiac hypertrophy compared to control mice transplanted with control kidneys, supporting a pivotal role for Ang II within the kidney in the development of target-organ damage.[159]

IV. Summary

In the first manuscript, we aimed to define the role of tubular NF-κB activation in AKI. First, we determined the time course of NF-κB activation in the kidney during ischemic AKI using a noninvasive in vivo imaging technique and a luciferase-dependent NF-κB reporter mouse model (κ-Luc). Using this mouse model and detection method, we found a significant increase in NF-kB activity as soon as twelve hours after ischemic AKI induction. The activation lasted five days until it returned to baseline levels. The role of renal tubular NF-κB signaling in ischemic AKI was addressed by generating a new mouse model where NF-κB was tubular epithelial-specifically inhibited (Emx1-ΔN). Our results using this mouse model indicate that renal tubular epithelial NF-kB activation aggravates tubular injury and supports a maladaptive inflammatory response in renal ischemic injury.

The second work shed light on the pathophysiological role of the enzyme CTH, which is partly responsible for the gasotransmitter H_2S production in the kidney, in AKI using mice lacking CTH ($Cth^{-/-}$) as well as heterozygous and wild-type littermates. Systemic loss of CTH in the mouse model we used led to a 50% reduction in renal tissue H_2S levels. Still, it did not influence the immediate consequences of ischemic AKI, such as renal tissue damage, inflammatory cell infiltration, or expression of cytokines, chemokines, and adhesion molecules. However, others found opposing results using a different mouse linage, still targeting the Cth gene. Therefore, future studies are granted to clarify the role of CTH in AKI.

In the third study, we aimed to test the hypothesis that inhibition of TRPC6 may ameliorate the immediate outcome of AKI. We used genetically modified mice lacking Trpc6 (Trpc6-/-) and applied two orally active and selective TRPC6 inhibitors, SH045 and BI-749327. We applied our well-established ischemic AKI-induced renal damage model with two different ischemia times to mimic a moderate and a severe ischemic injury. We also measured the renal tissue concentration of SH045 after intravenous application and at the end of the AKI-induced damage. The measured concentrations were in the pharmacologically effective range. Despite our various approaches (including the genetic mouse model), we found no effect of lacking or inhibiting TRPC6 on the immediate outcome of AKI.

In the fourth study, we investigated the role of Th1 and Th17 immune cells in the development of Ang II-induced organ damage. We used here established KO mouse model for investigating the Th1 cell population (IFNγR KO) with appropriate control mice and neutralizing antibody treatments to interfere with Th17 cell production (IL-23R antibody) or function (IL17A antibody). We concluded from this study that interfering with Th1 cell function by deleting the IFNγR protects from Ang II-induced cardiac damage and electric remodeling, whereas interfering with the Th17 pathway by IL23R or IL17 antibody treatment does not. On the other hand, in the kidney, IFNγ signaling acts in a cell type-specific manner. Renal function, cellular infiltration, and expression of epithelial damage markers were ameliorated in the absence of the IFNγR; however, it led to podocyte damage and loss, suggesting that IFNγR signaling is necessary to maintain normal podocyte integrity and function in the presence of Ang II.

In the fifth work, we investigated the role of signalosome bridging molecule Bcl10, which mediates AT1R signaling to NF-κB signaling in developing Ang II-induced renal damage. We used Bcl10 KO and control mice and performed allogeneic kidney transplantation to disentangle the role of Bcl10 in the kidney from lacking Bcl10 globally. We found that lacking Bcl10 ameliorates Ang II-induced cellular infiltration and renal fibrosis but surprisingly aggravated albuminuria. Allogeneic kidney transplantation of Bcl10 KO or control kidneys in control recipients revealed that Bcl10 is required for the correct adaptation of podocytes in an Ang II-induced hypertension model and uncovered unexpected implications regarding Ang II-induced cardiac hypertrophy.

V. Literature

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Erklärung

§ 4 Abs. 3 (k) der HabOMed der Charité

Hiermit erkläre ich, dass

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